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**³²P-UPTAKE BY DORSAL AND VENTRAL
HALVES OF THE IRIS AT THE BE-
GINNING OF LENS REGENERA-
TION IN THE NEWT***

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It is well-known as Wolffian lens regeneration that when a lens is completely removed from the eye of the newt, a new lens is formed from the mid-dorsal margin of the iris. From the standpoint of experimental morphology, SATO (1930) and MIKAMI (1941) made careful studies on lens regeneration in the newt, carrying out several experiments to analyse the causal mechanisms underlying the process. According to their findings, there is a gradient in the capacity for lens regeneration, and the potency is highest at the pupillary margin of the mid-dorsal iris and it declines gradually to the opposite ventral part.

As the first effort to find out biochemical processes which may prove the lens potency and its gradient in the iris, the phosphorus metabolism of the iris in the course of lens regeneration was investigated in the present study.

MATERIAL AND METHODS

The experiments were performed using adults of the newt, *Triturus pyrrhogaster*. The lens was removed from both eyes by the usual method (SATO 1930). Immediately after, and 2, 4 and 6 days after lens-removal, 0.5 ml. of TYRODE solution for amphibia

* The present experiment was performed at the Sugashima Marine Biological Station of Nagoya University. The authors wish to express their cordial gratitudes to Dr. MASAO SUGIYAMA, the director of the station, for his kind support to the present study. Also they gratefully acknowledge their indebtedness to Prof. Dr. TADAO SATO for his advice and suggestions in the course of the work

containing 1.6 μ C. of ^{32}P -orthophosphoric acid was injected into the body cavity. Animals thus treated were kept at room temperature of 15° to 20°C. for 48 hours. The number of newts used in each experimental series was 70 to 90. As the control, 90 animals without lens-removal were treated with the same ^{32}P solution. The radioactivity of the iris was measured after it had been separated into dorsal and ventral halves. The separation was carried out in RINGER's solution. Each half of the iris was homogenated in the RINGER's solution of pH 7.1 after careful washing with the same solution.

For the measurement of *radioactivity*, each sample was digested with 60% HClO_4 , and after neutralization with ammonia 0.3 ml. of KH_2PO_4 solution (1 mg. P/ml.) was added as carrier. KH_2PO_4 solution (1 mg. P/ml.) was added as carrier. The precipitate was obtained by adding FISKE and SUBBAROW reagent (10% CaCl_2 in saturated $\text{Ca}(\text{OH})_2$ solution). The activity of ^{32}P in the precipitate, was measured by means of the GEIGER-MÜLLER counter. *Totalphosphorus* was determined by the method of ALLEN (1940), and the determination of *total nitrogen* was made colorimetrically by adding NESSLER's solution after the homogenates had been digested in H_2SO_4 .

EXPERIMENTAL RESULTS

1. ^{32}P -uptake by the iris of the intact eye

48 hours after injection of ^{32}P -phosphoric acid into the body cavity of the newt without lens-removal, the radioactivity both in dorsal and in ventral iris was measured. Some of the results are shown in Table 1.

No difference in the amount of total phosphorus between dorsal and ventral halves was detected.* Furthermore, no significant morphological difference between both halves could be observed, except in the rudiment of the fetal choroid fissure of the ventral side of the iris. As shown in Table 1, the uptake of ^{32}P in the dorsal half was higher than that in the ventral one. These facts seem to imply that phosphorus metabolism is more active in the dorsal part than in the ventral, even when the lens is intact.

* The amount of total P calculated in the ratio of P to N was $270 \pm 25 \gamma/\text{mg. N}$ in both halves.

Table 1. Radioactivities with reference to total N and total P in dorsal and ventral halves of the iris of the eye without lens removal

	Experiment No.			
	1*		2	
	c.p.m./mg. N	c.p.m./mg. P	c.p.m./mg. N	c.p.m./mg. P
Dorsal half of iris	204	819	79	304
Ventral half of iris	139	590	44	182
Ratio (Dorsal/Ventral)	1.47	1.39	1.80	1.67

* In the experiment No. 1 the activity of ³²P-phosphoric acid injected was 3.2 μ C/0.5 ml. (2 times that injected in other experiments).

2. Changes of ³²P-uptake by the iris in the early stages of lens regeneration

Radioactivities were respectively measured in dorsal and in ventral halves 2, 4, 6 and 8 days after lens-removal. These results are summarized in Table 2, together with the data of the normal iris of the control eye.

Table 2. Radioactivities of dorsal and ventral halves of the iris referred to total N and total P in the course of lens regeneration

Days after lens removal	c.p.m./mg. N			c.p.m./mg. P		
	Dorsal half of iris	Ventral half of iris	Ratio (d/v)	Dorsal half of iris	Ventral half of iris	Ratio (d/v)
0(Control)	79	44	1.80	304	182	1.67
2	112	112	1.00	447	425	1.05
4	192	131	1.47	551	459	1.27
6	222	92	2.42	760	324	2.34
8	488	69	7.08	1221	204	5.99

Whereas the radioactivity of the dorsal iris with reference to both total nitrogen and total phosphorus steadily increased after lens-removal, the ventral iris showed a rapid increase of the activity at the early stage (up to the 4th day after lens-removal), which then decreased towards the level of the control iris. These changes are indicated in the graphs of Figs. 1 and 2. In order to compare these figures with morphological changes of the iris after lens-removal, some photographs are given in Fig. 1. Up to the 4th day

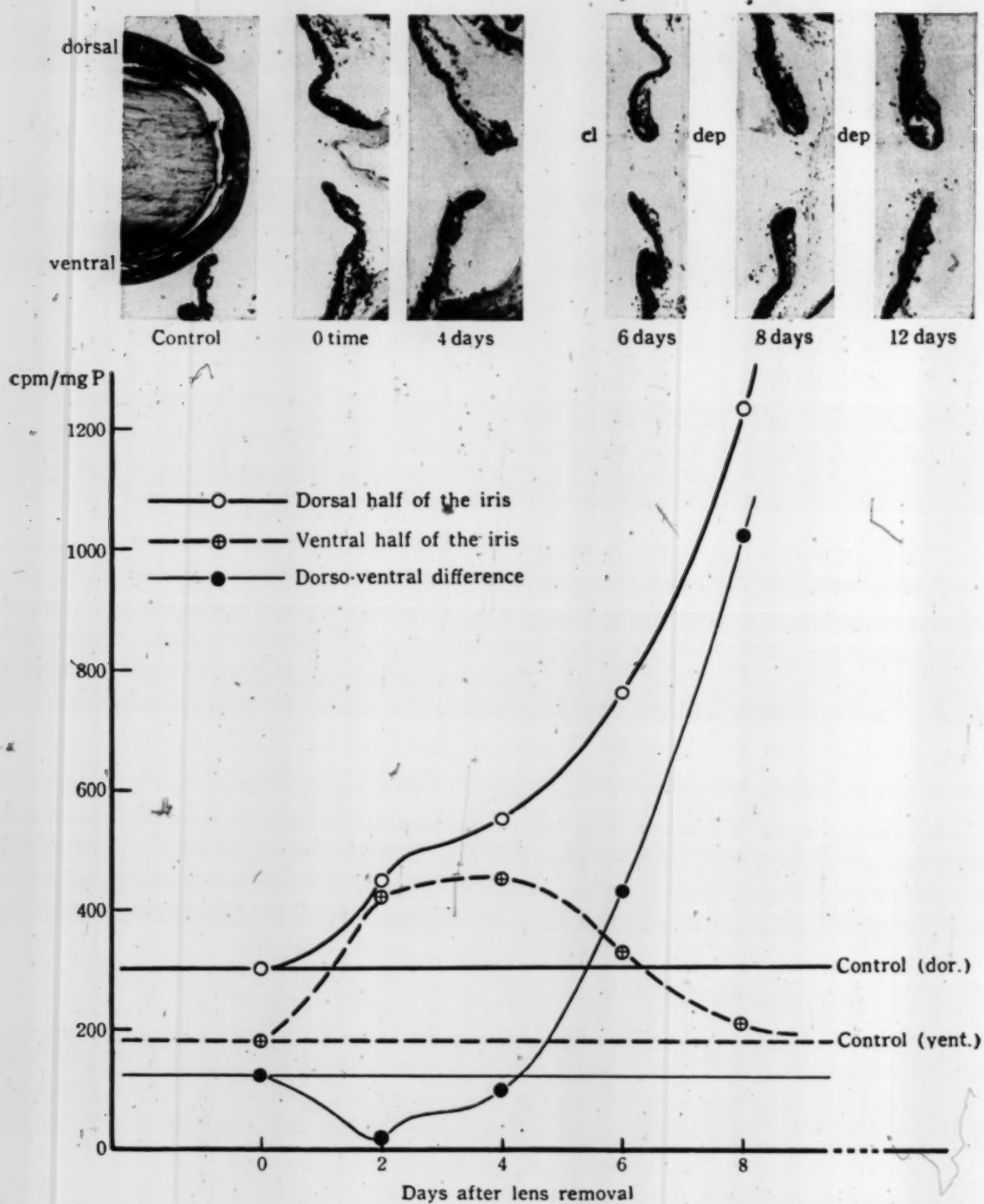


Fig. 1. Changes of the radioactivity referred to total phosphorus during early stages of lens regeneration.

Photographs show morphological changes of the iris.

cl: cleft dep: depigmented part

after lens-removal, morphological changes of the iris were not significant. On the 5th or the 6th day, thickening of the dorsal part of the iris and a cleft between the inner and outer lamellae were

observed. Within the 7th to the 8th day, first appearance of depigmentation occurred at the pupillary margin of the mid-dorsal part of the iris.

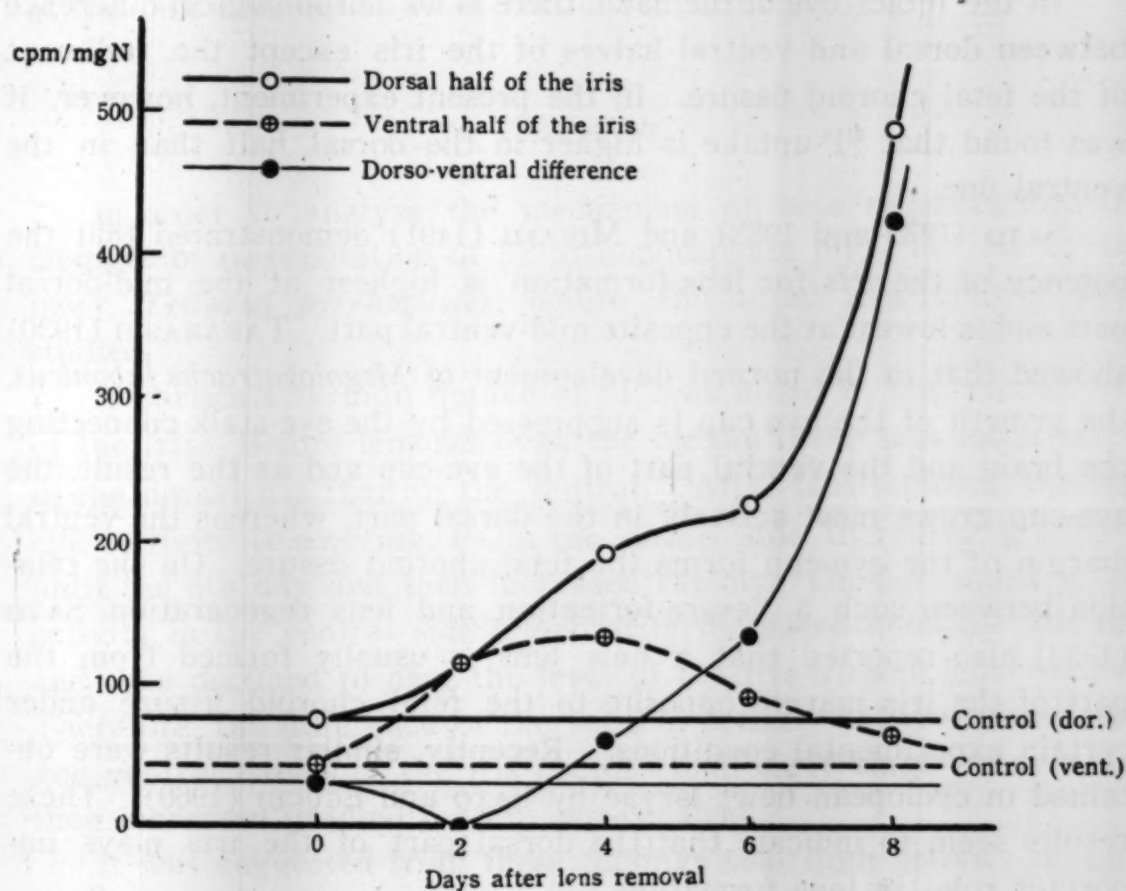


Fig. 2. Changes of the radioactivity referred to total nitrogen during early stages of lens regeneration.

As the increase of ^{32}P -uptake was shown in both halves in the early period, no remarkable difference of the uptake between both halves was recognized in the corresponding period, which was called the latent period of lens regeneration by REYER (1954). After this period, the difference became conspicuous in the initial period of lens regeneration (called by REYER 1954).

It is worthy to note that the ^{32}P -uptake by the dorsal iris shows a marked increase between the 6th and the 8th day, while the activity in the ventral iris shows gradual decline from the 4th day and nearly reaches the level of the control iris by the 8th day.

Therefore the ratio of the dorsal activity to the ventral is about 6-7: 1 on the 8th day.

DISCUSSION

In the intact eye of the newt, there is no morphological difference between dorsal and ventral halves of the iris except the rudiment of the fetal choroid fissure. In the present experiment, however, it was found that ^{32}P -uptake is higher in the dorsal half than in the ventral one.

SATO (1930 and 1933) and MIKAMI (1491) demonstrated that the potency of the iris for lens-formation is highest at the mid-dorsal part and is lowest at the opposite mid-ventral part. TAKAHASHI (1930) showed that in the normal development of *Megalobatrachs japonicus*, the growth of the eye-cup is suppressed by the eye-stalk connecting the brain and the ventral part of the eye-cup and as the result, the eye-cup grows most actively in the dorsal part, whereas the ventral margin of the eye-cup forms the fetal choroid fissure. On the relation between such a fissure-formation and lens regeneration, SATO (1938) also reported that a new lens is usually formed from the part of the iris margin opposite to the fetal choroid fissure under certain experimental conditions. Recently, similar results were obtained in cyclopean newt larvae by SATO and EGUCHI (1960). These results seem to indicate that the dorsal part of the iris plays important roles in lens formation.

Under the present experiment, the first sign of the lens regeneration began to appear about on the 7th day as described before. The present research, therefore, shows that increase of the ^{32}P -uptake takes place before appearance of the morphological changes of the iris. The rapid ascent and relapse of the specific activity (c.p.m./mg. P) of the ventral iris, which occur in the early period of lens regeneration might be conceived as the result of a temporary change of the physiological state of the iris being due to the operative stimuli.

The rapid increase of ^{32}P -uptake of the dorsal half was occurred at the 6th to the 8th day after lens-removal, whereas ^{32}P -uptake of the ventral half decreased nearly to the level of the control iris in the same period. These results suggest the possibility that rapid

increase of phosphorus metabolism is associated with the depigmentation of the dorsal pupillary margin and the lens forming potency of the dorsal iris.

The identification of phosphorus compounds accumulated in the dorsal iris as well as the elucidation of the biosynthetic mechanism of the lens fiber must await further experiments.

SUMMARY

In order to analyse the mechanism of lens regeneration, the changes of incorporation of ³²P-orthophosphate into the iris of the newt, *Triturus pyrrhogaster*, before and after lens-removal were studied.

A particularly high uptake of ³²P was noted in the dorsal half of the iris. Before lens-removal, the uptake of ³²P was more active in the dorsal iris than in the ventral. After lens-removal, the specific activity (c.p.m./mg. P) in the dorsal side increased gradually until the 6th day and then increased rapidly. On the contrary, the activity in the ventral side temporarily increased until the 4th day and then declined to near the level of the iris without lens-removal. Therefore, the difference of the specific activity between the dorsal and ventral halves of the iris decreased in early stage (2 days), and then increased gradually after lens-removal.

It was suggested from these findings that high activity of phosphorus metabolism in the dorsal side of the iris after lens-removal is partly associated with the process of the formation of a new lens.

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ON THE NATURE OF AMPHIBIAN PHENOL OXIDASE

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INTRODUCTION

Rapid progress has been made in the analysis of the mechanism of melanin formation in vertebrates since tyrosinase and dopa oxidase were detected in mammalian melanoma tissues. In various non-amphibian vertebrates tyrosinase activity was demonstrated only in embryos, fetuses and melanomas (cf. DAWSON and TARPLEY, 1951; LERNER, 1953; MASON, 1955). In amphibians, BAKER (1951, 1953) analyzed the relation between certain genes and phenol oxidase activity in *Rana pipiens*.

Embryonic materials are well suited for the study of the nature of phenol oxidase, particularly during its synthesis and the establishment of its specificity.

In the present paper melanin formation in amphibia is studied and is discussed from an embryological viewpoint.

MATERIAL AND METHODS

The materials used in the present investigation were embryos, larvae and adults of the newt (*Triturus pyrrhogaster*), two species of the frog (*Rana nigromaculata* and *Rhacophorus schlegelii*) and the toad (*Bufo vulgaris japonicus*). Whole embryos, larvae with the internal organs removed, and isolated pieces of adult skin were homogenized in distilled water in a glass homogenizer of the ordinary type. The homogenates were centrifuged at 3000 r.p.m. for 10 minutes. The supernatant was used as the crude enzyme solution. When necessary the enzyme solution was further fractionated by adding ammonium sulfate equivalent to 30-60% saturation.

¹⁾ The author wishes to express his sincere gratitude to Prof. TOKI-O YAMAMOTO for the constant guidance and encouragement throughout the course of the present experiments.

Enzymatic activity was measured by means of a Warburg manometer, and expressed in terms of oxygen consumption at 30°C. Parallel observations were made on the color changes of reacting solutions. The main part of the vessel contained 1 ml. of enzyme solution and 0.5 ml. of M/10 buffer solution. The side arm contained 0.5 ml. of a substrate and the center well 0.1 ml. of 20% potassium hydroxide. A parallel control series run without substrate provided the correction for endogenous respiration. When tyrosine and dopa were used as substrate, 0.1 ml. of M/10 moniodoacetic acid or M/10 moniodoacetamide was added in order to reduce endogenous respiration. These reagents hardly showed any effect on enzymatic activity. The autoxidation of dopa proved to be negligible during several hours at a pH below 6.8.

The following reagents were used:

Substrate stock solution: 0.1% *D, L*-tyrosine solution in 0.08% sodium carbonate, 0.1% *D, L*-dopa (3,4-dihydroxyphenylalanine), 0.5% *p*-, *m*- and *o*-cresols, 0.5% phenol, 0.5% *p*- and *m*-nitrophenols, 0.5% *p*-chlorophenol, 0.5% catechol, 0.5% bromophenol, 0.5% 3,4-dimethylphenol, 0.5% 3,5-dimethylphenol and 0.5% 3,4-dihydroxybenzoic acid. The stock solutions were diluted to appropriate concentrations before use.

Inhibitors used were 10^{-2} – 10^{-6} M sodium cyanide, 10^{-1} – 10^{-5} M thiourea, 10^{-3} – 10^{-7} M phenylthiourea, 10^{-3} – 10^{-6} M diethylthiocarbamate, 10^{-2} – 10^{-6} M thiouracil, and 10^{-4} – 10^{-7} M 8-oxyquinoline. Buffer solutions employed were M/10 phosphate buffer, M/10 acetate buffer and M/10 veronal buffer. Cupric sulfate solutions (10^{-3} – 10^{-7} M) were used both for eliminating the latent period and for reactivating the copper-free enzyme.

The histochemical test for phenol oxidase was carried out as follows: a piece of skin was immersed in a medium consisting of three parts of a substrate solution (tyrosine, dopa, *p*-cresol or *p*-chlorophenol) and two parts of phosphate buffer (pH 6.8) at 30°C. When the medium became colored, it was renewed.

In the following pages, enzymes are named according to the respective substrate they oxidize (e.g. "*p*-cresol oxidase", "*p*-chlorophenol oxidase").

Further details of experimental procedures are given in the relevant sections.

RESULTS

a) The nature of phenol oxidase

Newt skin was homogenized in distilled water, in $m/8$ sodium chloride or in $m/50$ phosphate buffer, and the homogenate was centrifuged at 3000 r.p.m. for 20 minutes. The precipitate, containing melanin, showed little *p*-cresol oxidase activity. Most of the phenol oxidase activity was found in the supernatant. Ammonium sulfate was added to the supernatant (30% saturation) and the precipitate was discarded. Ammonium sulfate was further added to the solution (60% saturation) and the precipitate containing the major part of the total phenol oxidase activity was obtained. The combined precipitate obtained with ammonium sulfate outside the limits of 30 to 60% saturation, *i.e.* lower than 30% and higher than 60%, showed only about 20% of the total activity. Amphibian monophenol oxidase was characterized by a latent period. The duration of the latent period depended largely upon the degree of purification of the enzyme and on the source material. It is not always correlated with the enzymatic activity nor with the pH of the reacting solution in question. It could be shortened by addition of copper ions or diphenols. Monophenol oxidase preparations fractionated with ammonium sulfate were characterized by a short latent period.

b) Substrate specificity

The phenol oxidase of newt skin could oxidize *p*- and *m*-cresols, phenol, *p*-chlorophenol, *p*-bromophenol, catechol, tyrosine and dopa, but it could not oxidize 3,4-dimethylphenol, 3,5-dimethylphenol, *p*- and *m*-nitrophenols, 3,4-dihydroxybenzoic acid, and *o*-cresol.

The phenol oxidase of frog skin showed the same substrate specificity as that of newt skin. However, the phenol oxidase of tadpole could oxidize only tyrosine and dopa.

c) Localization in the body

The dorsal skin of the newt is rich in melanin pigment, whereas the ventral skin contains little melanin. Extracts of dorsal skin showed a phenol oxidase activity which was nearly twice as high as that of ventral skin extracts. Histochemically, no ("amelanotic") melanophores could be detected in the ventral newt skin, with tyrosine, dopa or *p*-cresol as substrate, although ventral skin ex-

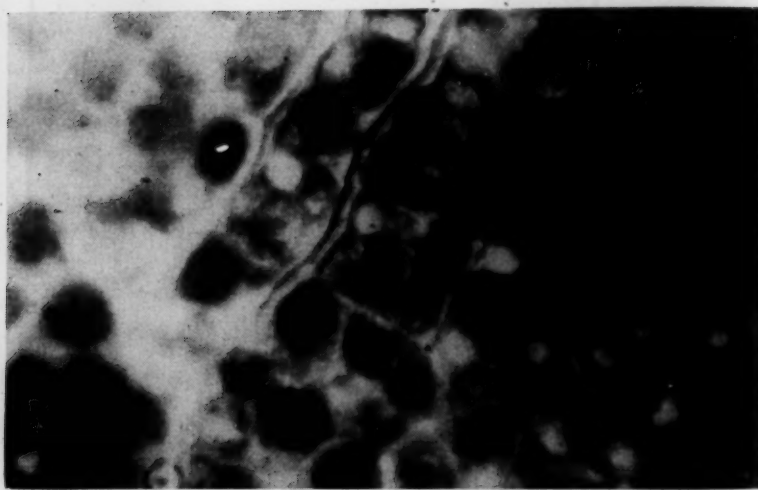
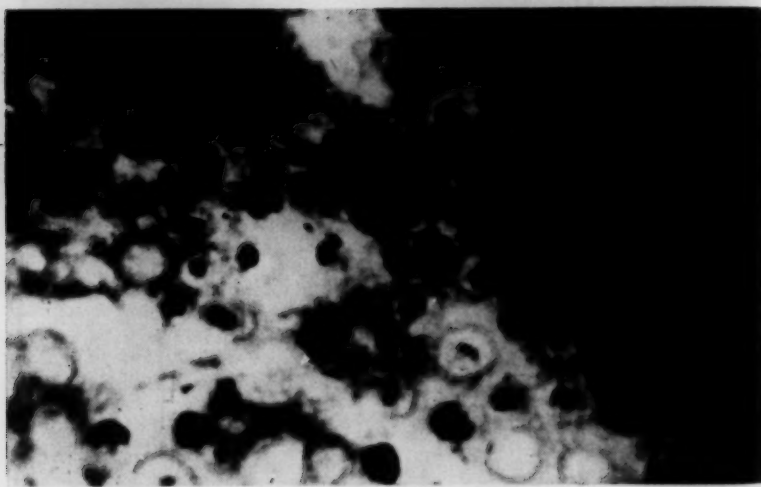
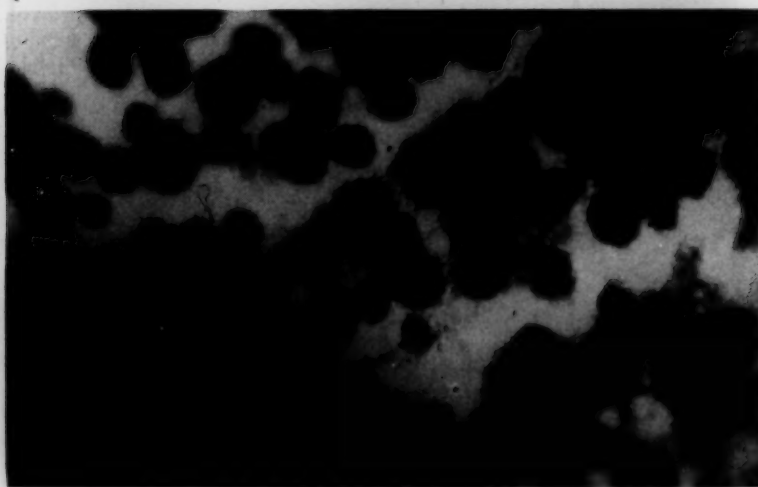
*a**b**c*

Fig. 1. Ventral skin of the newt (*Triturus pyrrhogaster*). \times ca. 40.

a. Normal untreated condition.—*b.* Tyrosinase test. Note the black pigment locating in skin glands and epithelial cells.—*c.* *p*-cresol oxidase test. Note the red pigment locating in skin glands and epithelial cells.

tracts contained phenol oxidase. Black or red pigment resulting from the oxidation of tyrosine, dopa or *p*-cresol were found in the skin glands and in various parts of the epithelium. These facts suggest that phenol oxidase is not always bound to melanin granules in the newt skin. The results are shown in Table 1 and Figures 1 *a-c*.

Table 1. Phenol oxidase activity of dorsal and ventral newt skin

	Tyrosinase	<i>p</i> -cresol oxidase
Dorsal (black region)	10-15	50-70
Ventral (red region)	5-9	33-50

Activity is expressed as oxygen consumption per hour per standard amounts of skin extract (prepared from 200 mg. wet weight of skin) in the presence of substrate. Endogenous oxygen consumption is subtracted.

The dorso-ventral distribution of melanin in the frog skin was similar to that in the newt. Extracts of dorsal skin showed high phenol oxidase activity, whereas extracts of ventral skin showed very low activity. Histochemically, no "amelanotic" melanophores could be detected in the ventral frog skin.

Homogenates of dorsal skin contained many melanin granules. They were not precipitated by centrifuging at 3000 r.p.m. for 30 minutes. After shaking with chloroform, however, the granules were easily precipitated by weak centrifugal forces. Phenol oxidase activity was present in the supernatant.

Phenol oxidase was not detected in internal organs such as liver, kidney, ovary, muscle, etc.

d) Effect of pH on phenol oxidase activity

In this series of experiments, the substrates used were *p*- and *m*-cresols, *p*-chlorophenol, *p*-bromophenol, tyrosine and dopa. Phenol oxidase was inactivated at pH value below 4.5. Above pH 5.0 particularly in the range from 5.5 to 7.5 the activity was nearly constant, and there was no distinct pH optimum. The results are shown in Figure 2.

By the addition of copper ions, phenol oxidase of the newt was

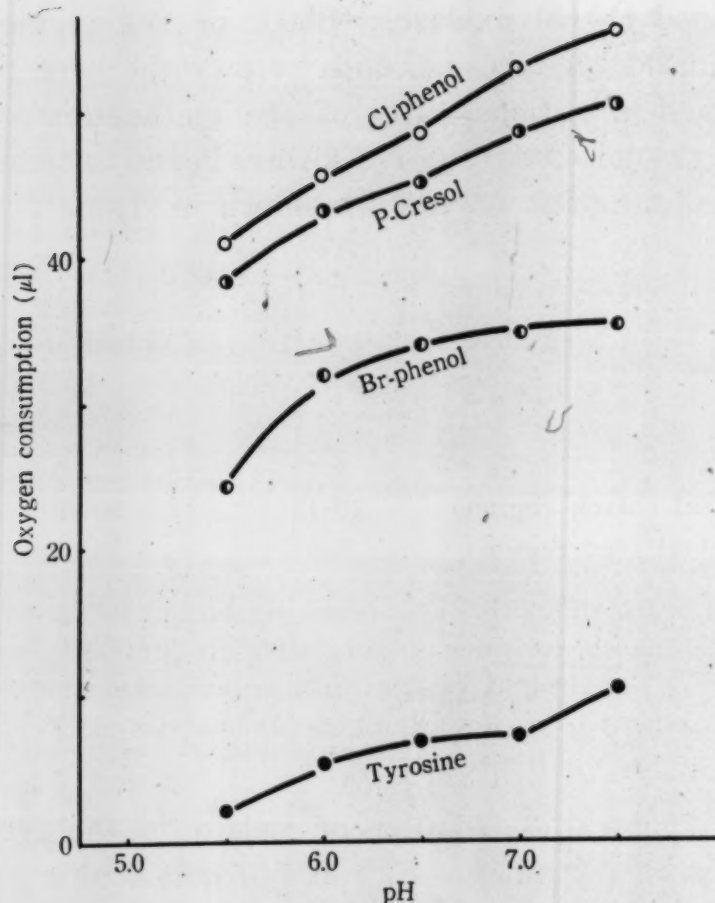


Fig. 2. The relation between phenol oxidase activity and pH. Enzymatic activity is expressed as oxygen consumption per 15 minutes for a standard amount of extract. Endogenous respiration is subtracted.

activated and its latent period was eliminated. Copper ions, however, had no influence on the pH effect.

At pH values above 7.5, polymerization of autoxidized substrate is accelerated to such an extent that true enzymatic activity can no longer be measured.

e) Effect of metal ions

In order to remove copper, newt skin homogenate was treated with $M/50$ sodium cyanide for 24 hours, followed by dialysis in the cold for 24 hours against $M/100$ phosphate buffer of pH 6.8. By this treatment phenol oxidase activity was lost, but it was recovered on addition of copper ions.

Other metal ions such as Fe, Ni, Co, Mn, Zn and Hg could not

replace copper, and they had no influence on the length of the latent period of monophenol oxidase.

Tyrosinase was activated by 10^{-3} M copper, but the activity of *p*-cresol oxidase was inhibited at this concentration. The extent of activation by copper depended on the kind of substrate used.

f) Effect of temperature

The activity of newt enzyme was measured at 20° and 30°C. with *p*-cresol, *p*-chlorophenol and *p*-bromophenol as substrate.

The Q_{10} of phenol oxidase was found to be 1.1-1.3, differences according to the substrates being negligible. When copper ions were added, Q_{10} was increased to 1.3-1.5. The results are shown in Table 2.

Table 2. Temperature quotient of phenol oxidase and effect of copper ions on it

Enzyme	Copper ion concentrations	Q_{10}
<i>p</i> -cresol oxidase	no copper	1.1-1.3
	5×10^{-7} M	1.0-1.3
	5×10^{-6} M	1.3-1.4
	5×10^{-5} M	1.5-1.8
	5×10^{-4} M	1.4-1.7
<i>p</i> -chlorophenol oxidase	no copper	1.1-1.2
	5×10^{-5} M	1.2-1.4
	5×10^{-4} M	1.2-1.5
<i>p</i> -bromophenol oxidase	no copper	1.1-1.2

g) Effect of copper-binding inhibitors

If the various phenol oxidase activities were exerted by the same copper protein, copper-binding substances would inhibit the reactions with different substrates to the same extent. In amphibians, phenylthiourea inhibited *p*-cresol oxidase and *p*-chlorophenol oxidase to the same extent, but tyrosinase and dopa oxidase were inhibited to different extents, tyrosinase being more sensitive. Similar results were obtained with other inhibitors such as diethylthiocarbamate, thiourea and sodium cyanide. The results are shown in Table 3.

h) Developmental study of melanin formation

Except in the *Rhacophorus*-egg, the embryonic melanin pigment is

Table 3. The concentration of inhibitors needed for 50% inhibition of phenol oxidase activity

Inhibitors	Tyrosinase	<i>p</i> -cresol oxidase
Phenylthiourea	10^{-5} – 10^{-6} M	10^{-4} – 10^{-5} M
Sodium cyanide	10^{-4} – 10^{-5} M	10^{-3} – 10^{-4} M
Thiourea	10^{-3} – 10^{-4} M	10^{-1} – 10^{-2} M
Thiouracil	10^{-4} – 10^{-5} M	10^{-2} – 10^{-3} M
Diethylthiocarbamate	10^{-4} – 10^{-5} M	10^{-3} – 10^{-4} M
8-oxyquinoline	10^{-5} – 10^{-6} M	10^{-4} – 10^{-5} M

present in the eggs of all the other species used. The embryonic pigment exhibited no phenol oxidase activity. In the toad, tyrosinase and dopa oxidase activities appeared during the differentiation of melanophores, and increased in the course of development. As mentioned above, other phenol oxidase activities appeared only after metamorphosis. The results are shown in Table 4.

Table 4. Phenol oxidase activity during development in the toad

Stage	Tyrosinase	Dopa oxidase	<i>p</i> -cresol oxidase
Neurula	—	—	—
Tailbud	—	—	—
Tadpole (not swimming)	—	—	—
Tadpole (swimming)	—	1–2	—
Tadpole (with hind limb buds)	1.5	4	—
Tadpole (with early hind limbs)	2.5	12	—
Tadpole (with hind limbs)	3.0	12	1
Tadpole (with fore limbs)	5.0	10	4

Activity is expressed as oxygen consumption per hour of 10 animals, in the presence of substrate. Endogenous oxygen consumption is subtracted.

The fate of the embryonic melanin is not yet clear. In the tadpole of the toad, prior to the differentiation of epidermal melanophore, large rod-shaped cells appeared in the epidermis in which the embryonic melanin granules were concentrated. They may perhaps not be regarded as precursors of melanophores, because they are also present in tadpoles cultured in $M/3,000$ phenylthiourea

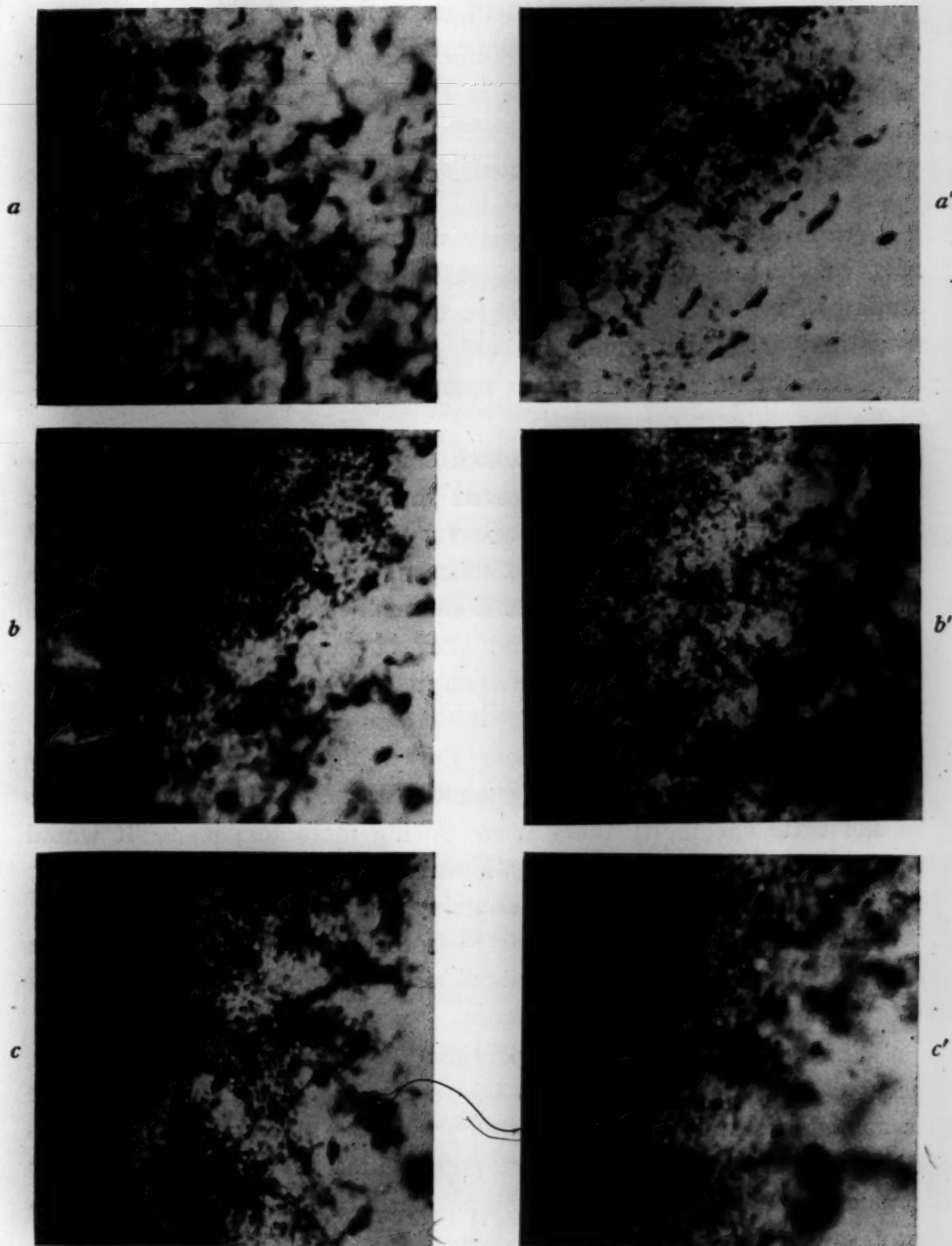


Fig. 3. Epidermis of toad tadpole (*Bufo vulgaris japonicus*). $\times ca. 75$.
 a-c: Normal condition.—a. From 13 mm. tadpole. Note rod-shaped cells.
 —b. From 17 mm. tadpole. The rod-shaped cells are disappearing and epidermal melanophores begin to differentiate.—c. From 18 mm. tadpole. Note epidermal melanophores.
 a'-c': After treatment with M/3,000 phenylthiourea.—a'. From similar tadpole as a. Note rod-shaped cells.—b'. From 15 mm. tadpole. Note the rod-shaped cells are disappearing, but epidermal melanophores do not differentiate.—c'. From similar tadpole as c. Epidermal melanophores are absent, a few rod-shaped cells are remaining.

although the melanin formation in the cells is inhibited in the solution. The rod-shaped cells disappeared in the course of development (See Figures 3, *a-c*, *a'-c'*).

DISCUSSION

While phenol oxidase of mammals only catalyzes the oxidation of tyrosine and dopa (LERNER, 1953), phenol oxidase from adult amphibians oxidizes not only these two but also other phenols such as *p*- and *m*-cresols, *p*-chlorophenol, *p*-bromophenol, phenol, catechol, etc. Amphibian phenol oxidase resembles that of plants and insects in its low substrate specificity.

In the newt, ventral skin which contained neither melanin nor "amelanotic" melanophores, showed phenol oxidase activity. This fact constitutes indirect evidence for the assumption that phenol oxidase activity *in vivo* may be concerned with functions other than melanin formation. BAKER (1953) determined tyrosinase activity in pigmented spots and background skin in *Rana pipiens*, and found that there is no correlation between melanin content and tyrosinase activity.

Phenol oxidase from tadpole shows high substrate specificity similar to that of mammalian phenol oxidase. A profound change in substrate specificity takes place during metamorphosis. It would be interesting to know whether larval enzyme systems persist in the adult or they are replaced by adult systems.

MASON (1948) made a spectrophotometric analysis of melanin formation in mammalian melanoma tissue, and found it to be similar to that in invertebrates and plants.

The important role of copper ions in amphibian phenol oxidase activity supports the view that in amphibians also phenol oxidase is copper-protein. The Q_{10} of phenol oxidase activity shifted toward higher values in the presence of copper ions. It may be possible to consider that copper ions combine with protein to form copper-protein with an enzymatic activity.

It is an important question whether phenol oxidase is a single protein with various activities or a group of proteins, each having its own substrate specificity. LERNER *et al.* (1948) have shown that tyrosinase and dopa oxidase of mouse melanoma cannot be fraction-

ated by centrifugal forces, alcohol, acetone and ammonium sulfate. They concluded that tyrosinase and dopa oxidase are a single enzyme. In the present investigation it was shown that the effect of copper-binding inhibitors differs for various substrates, and that the substrate specificity changes in the course of development (particularly during metamorphosis). The conclusion may be drawn that amphibian phenol oxidase is a mixture of various phenol oxidases, activities of which *in vivo* are concerned with melanin formation as well as with other still unknown processes.

SUMMARY

1) Phenol oxidase from the skin of amphibians (*Triturus pyrrhogaster*, *Rana nigromaculata* and *Bufo vulgaris japonicus*) can oxidize phenols such as *p*- and *m*-cresols, phenol, *p*-chlorophenol *p*-bromophenol, tyrosine, dopa and catechol, but not *p*- and *m*-nitrophenols, 3,4-dimethylphenol, 3,5-dimethylphenol, 3,4-dihydroxybenzoic acid and *o*-cresol.

2) Amphibian phenol oxidase is probably a copper protein. Its activity is inhibited by phenylthiourea, sodium cyanide, diethylthiocarbamate and 8-oxyquinoline.

3) The Q_{10} of the enzyme is 1.1-1.3. In the presence of copper ions it increases to 1.3-1.5.

4) There is no distinct pH optimum. Activity is nearly constant at pH values ranging from 5.0 to 7.5.

5) The substrate specificity of the enzyme may change in the course of development.

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PHYSIOLOGICAL STUDY ON EGG FORMATION OF THE FISH

I. ACCUMULATION OF CARBOHYDRATES AND PROTEINS DURING OOGENESIS

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Up to the present, many studies have been done on the metabolism of carbohydrates and proteins during oogenesis. Most of the works in this field were concerned with the eggs of marine invertebrates and the hen, but comparatively little has been studied concerning the fish egg. For this reason, the author planned to study the accumulation of carbohydrates and proteins during oogenesis of the fish egg in order to elucidate the material base of the egg formation in the lower vertebrates.

MATERIALS AND METHODS

Materials used were eggs of an orange-red variety of the medaka, *Oryzias latipes*. Adult females lay eggs daily for a considerable period through spring and summer. For obtaining eggs, the method described by YAMAMOTO (1944) was adopted. After pithing, the fish was dissected in the isotonic RINGER's solution¹⁾ and the entire ovary was removed. Because each ovary contains ova in various phases of formation, the ovaries were cut open under a binocular microscope and the eggs were selected in groups according to the grade of transparency and the size which increase with the progress of oogenesis. Removing of follicle cells from the egg surface was done with a fine needle and forceps under a binocular microscope after eggs were dipped in absolute alcohol.

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¹⁾ The solution is composed of 100 parts M/7.5 NaCl, 2.0 parts M/7.5 KCl and 2.1 parts M/11 CaCl₂; the pH of this solution was adjusted to 7.3 with M/15 phosphate buffer.

Measurement of glycogen content. Glycogen content was measured by BOETTIGER's method. 10 or 20 eggs were put in 2 cc. of 30% KOH solution and boiled for 20 minutes, after which 2 cc. of distilled water was added, followed by 6 cc. of absolute alcohol. After sufficient shaking, the mixture was left standing for 1 hour, and then centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant was drained off and the precipitate was dissolved in 2 cc. of distilled water. 5 cc. of a mixture of 100 cc. glacial acetic acid and 60 cc. concentrated hydrochloric acid containing 3 grammes of diphenylamine was poured into the above solution, and the glycogen content was measured colorimetrically by comparison with the standard glycogen solution.

For the purpose of determining the content of water-soluble glycogen 20-40 oocytes were broken in distilled water²⁾ and extracted by warming in a water bath. After centrifuging at 1,500 r.p.m. for 5 minutes, the supernatant was used for the determination.

Measurement of total hexose and glycogen content. For the measurement of the total contents of hexose and glycogen DREYWOOD's anthrone method, described in detail by DREYWOOD (1949), MORSE (1947), MORRIS (1948) and VILES and SILVERMAN (1949), was followed. The essential points of this method are as follows: Several eggs are put into a test tube filled with 2 cc. of 1 N hydrochloric acid. After sealing the tube, it is heated for some 20 hours in boiling water. The entire content is then, transferred to a test tube, and 2 cc. of distilled water and 1 cc. of concentrated hydrochloric acid are added. Next is added 8 cc. of anthrone solution prepared by adding 0.2 grammes of anthrone to 100 cc. of concentrated sulfuric acid, and the solution is quickly mixed by stirring. After 10 minutes or more, the colour is measured either by an electrophotometer against a tube containing distilled water with reagent only or colorimetrically against glucose standards. Colour filter of 600 m μ (orange) is used in both cases.

Measurement of nitrogen content. Total nitrogen content was

²⁾ The pH of the water was preliminarily adjusted to 7.2 with M/15 phosphate buffer solution. The adjustment of the pH is essential, because if the extraction is performed in non-buffered distilled water, the data obtained show variations.

measured colorimetrically by using NESSLER'S reagent. Non-protein-nitrogen in the oocyte was also determined. 40 to 50 oocytes were put into 2 cc. of distilled water and slowly homogenized in a glass homogenizer; 10 cc. of 10% trichloroacetic acid solution was added to the homogenized suspension. After 30 minutes, the suspension was centrifuged for 10 minutes at 3,000 r.p.m. Thus obtained supernatant was used as the solution of non-protein-nitrogen.

The representative value for each series of experiments is the average of at least five readings. Most of the experiments were performed at 25-28°C., which are the optimal temperatures for normal development of the medaka.

RESULTS

Glycogen content. The glycogen contents of the oocyte both with and without follicle cells were measured. The results are graphically shown in Figure 1. The content of glycogen begins to increase remarkably 2 days before spawning. This increase is more conspicuous in the oocyte freed from follicle cells than in that having follicle cells, suggesting that the glycogen in the follicle cells de-

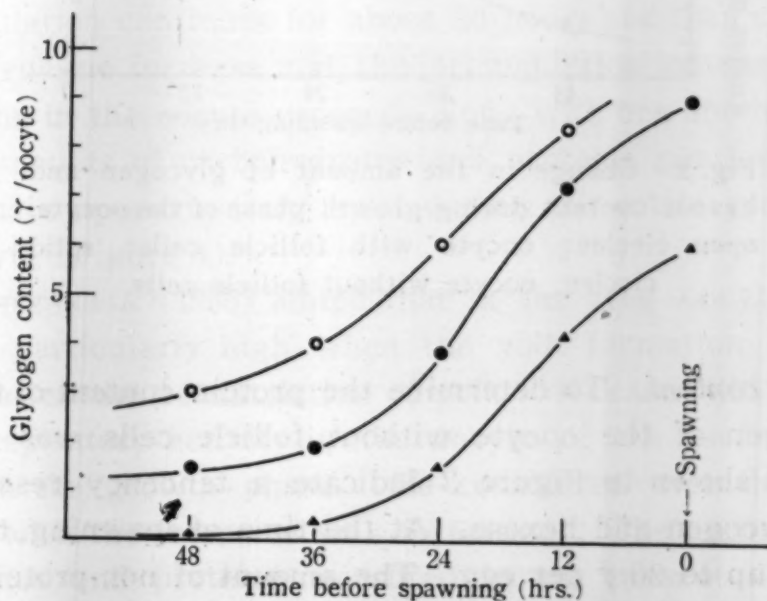


Fig. 1. Change in the glycogen content during growth phase of the oocyte.

open circles: glycogen of oocyte with follicle cells;
solid circles: glycogen of oocyte without follicle cells;
triangles: water-extractable glycogen.

creases gradually after the growth phase of the oocyte. Also for the water-extractable glycogen of the oocyte without follicle cells an increase was noted (Figure 1).

Total of hexose and glycogen content. Analyses of this series were carried out in oocytes during the growth phase. The results shown in Figure 2 indicate that the total amount of hexose and glycogen begins to increase conspicuously 2 days before spawning and reaches some 15 γ per egg at the time just before spawning.

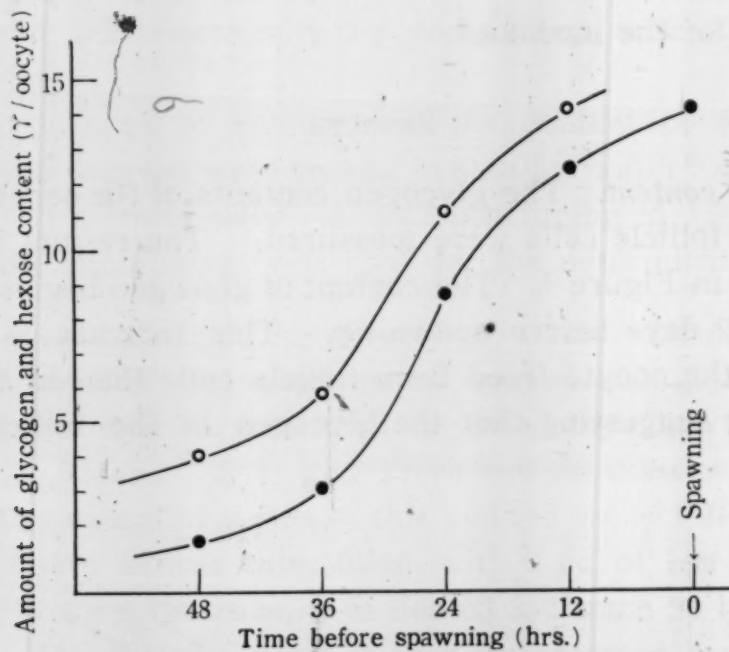


Fig. 2. Change in the amount of glycogen and hexose content during growth phase of the oocyte. open circles: oocyte with follicle cells; solid circles: oocyte without follicle cells.

Protein content. To determine the protein content of the oocyte, total nitrogen of the oocyte without follicle cells was measured. The results shown in Figure 3 indicate a tendency resembling to that for glycogen and hexose. At the time of spawning, total nitrogen comes up to 20 γ per egg. The amount of non-protein-nitrogen in the oocyte was also determined and was found always to be about one-tenth of the total nitrogen during the whole process of oogenesis. Therefore, the amount of nitrogen shown in Figure 3 roughly represents that of protein.

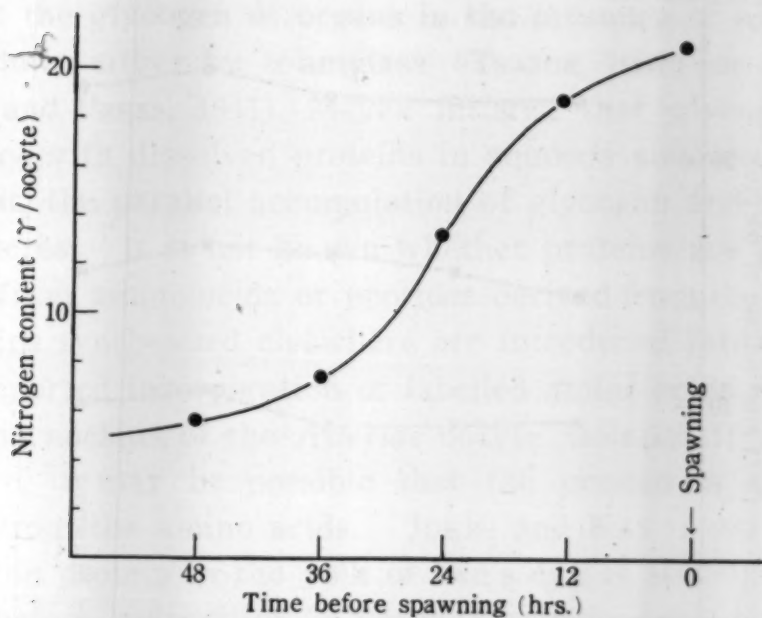


Fig. 3. Total nitrogen during growth phase of oocyte.

DISCUSSION

The volume of the oocyte of the medaka begins to increase about 48 hours before spawning. Just at that time large quantity of carbohydrates and proteins begins to accumulate in the oocyte and the accumulation continues for about 36 hours and then slows down. Thus, the volume increase and the accumulation of carbohydrates and proteins in the oocyte proceeds along with one another. Therefore, the amounts of carbohydrates and proteins per 1 mm³ of the oocyte remain approximately equal during the entire growth phase of the oocyte (Figure 4).

MESTCHRESKAJA (1935) stated that in the frog oocyte, the respiration is particularly high, when the yolk formation begins and then falls after the accumulation of yolk. NAKANO (1953) reported that the respiratory activity in the oocyte of the medaka rapidly increases in the growth phase. He considered that this may be associated with the intensive yolk formation. The accumulation of carbohydrates and proteins measured in the present study is in parallel with the increase of the respiratory activity of the medaka oocyte followed by a fall after the beginning of the maturation division (NAKANO 1953) which takes place about 10 hours before spawning. The present results suggest that the yolk formation

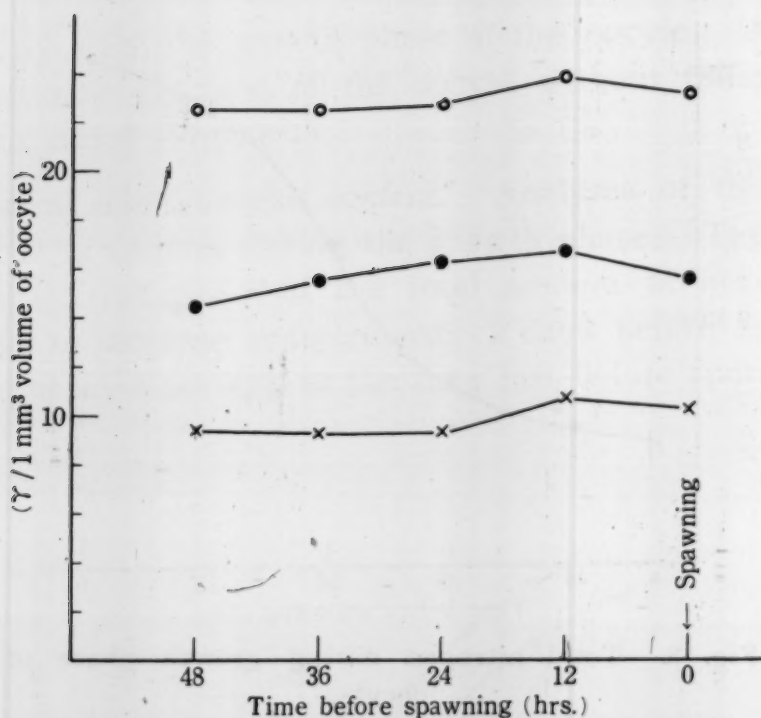


Fig. 4. Content of nitrogen (open circles), glycogen hexose (solid circles) and glycogen alone (crosses) in 1 mm³. of oocyte.

has almost been completed by 10 hours before spawning.

WILLSTÄTTER and ROHDEWALD (1934) reported that in living cells, two different states of glycogen can be distinguished, water-extractable and unextractable. It was insisted that the unextractable fraction is protein-bound "desmoglycogen", while the extractable fraction represents unbound "lyoglycogen". According to MEYER (1943), however, this difference is based only on a difference in the degree of polymerization of glycogen, the extractable glycogen being the low-polymeric fraction. WILLSTÄTTER and ROHDEWALD found in goose liver that the richer it was in glycogen, the greater the proportion of extractable glycogen, which they called "lyo-glycogen". This is also true in oogenesis of the medaka. It may be assumed that the glycogen which accumulates in the oocyte originates from the plasma. With accumulation of glycogen, hexose also accumulates in the oocyte.

The curve of accumulation of nitrogen resembles that of glycogen. As mentioned in "Results" (p. 242), the curve shown in Figure 3 roughly represents accumulation of protein-nitrogen. Based on the

fact that the glycogen of organs in the presence of albumin is not broken down either by α -amylase (TSAIOR, 1937) or by β -amylase (MEYER and PRESS, 1941), MEYER insisted that glycogen reacts in some way with dissolved proteins in aqueous solution. From this viewpoint, the parallel accumulation of glycogen and proteins is of some interest. It is not known whether proteins are synthesized in oocytes from amino-acids or peptides derived from the blood plasma, or proteins synthesized elsewhere are introduced into oocytes. FICQ (1955) reported incorporation of labelled amino-acids into the cytoplasm and nucleus of the *Asterias* oocyte isolated from the ovary. Therefore, it may be possible that the protein is synthesized in oocytes from the amino acids. JUKES and KAY (1932) stated that 99% of the protein in the yolk of hen's egg is secreted during 5 to 8 days before oviposition. NEEDHAM (1950) considered that this protein may have been produced from the amino-acids in the plasma or possibly from the plasma proteins by some other less drastic change.

SUMMARY

1. In the *Oryzias latipes*, changes of the contents of glycogen, hexose and proteins during growth period of oocyte were measured.
2. These substances accumulate noticeably at about 48 hours before spawning and continues for about 36 hours. After this period, the accumulation slows down.
3. While the increase of the water-extractable glycogen is remarkable during the growth period, that of the non-extractable glycogen is negligible.
4. In the amount per unit volume no change is observed for all of these substances during the whole period of oogenesis.

The writer expresses his gratitude to Prof. Dr. TOKI-O YAMAMOTO for his valuable suggestions and criticisms. Appreciation is due to Dr. EIZO NAKANO for his valuable suggestions.

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STUDIES ON THE ARTIFICIAL ACTIVATION AND CORTICAL REACTION OF THE EGG OF *NEREIS JAPONICA*

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The eggs of *Nereis japonica* are fertilized in brackish water which is equivalent to 70-80% sea water. After fertilization, the jelly layer is formed around the egg as the result of the cortical change. These processes are essentially similar to those of the eggs of other kinds of *Nereis* which were studied by LILLIE (1919), SPEK (1930) and COSTELLO (1939, 1948). The cortical layer of the *Nereis* egg is considered to contain the precursor of jelly substance, both the cortical layer and the jelly substance being stainable metachromatically with toluidine blue. The granules in the cortical layer are stainable especially in dark color, and disappear after the extrusion of jelly, the perivitelline space being also metachromatically stainable.

The author has performed some experiments on artificial activation of *Nereis* eggs in order to analyse the process of the cortical reaction and the formation of the jelly layer.

MATERIAL AND METHODS

The materials used were the eggs of *Nereis japonica* obtained from the Yoshino River in Tokushima City, Shikoku, in January and February. Eggs were shed and kept in 75% sea water. Solution of various reagents having the same osmotic pressure to that of 75% sea water were tested.

RESULTS

I. Activation of eggs with $KMnO_4$ and other oxidizing agents

It was reported by several workers that eggs of sea urchins and clams can be activated by $NaIO_4$ (RUNNSTRÖM and KRISZAT 1950, SAWADA 1955) and by $KMnO_4$ (MOTOMURA 1954). A question arose then if a similar result can be obtained in *Nereis* eggs.

In the first series of experiments, effects of periodate and some other oxidizing agents on the unfertilized egg of *Nereis* were investigated. In general, when the egg was activated, the germinal vesicle broke down and the polar bodies were frequently extruded, although further development did not ensue. It was found that 10^{-5} – 10^{-6} M KMnO_4 was effective for activation, while NaIO_4 not even at 10^{-2} M.

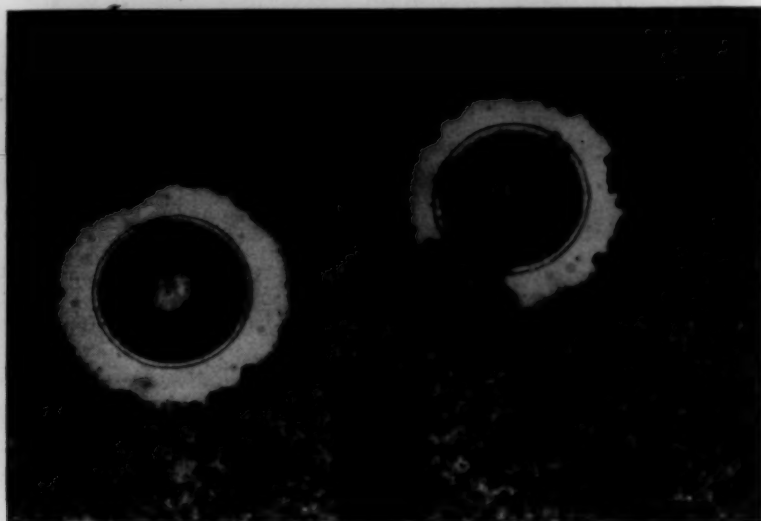


Fig. 1. Eggs activated by KMnO_4 (in Indian ink, $\times ca. 100$)

The length of time of immersion necessary for activation varied with the concentration of KMnO_4 : e.g., 10 minutes in 1×10^{-6} M, 3 minutes in 1×10^{-5} M, and less than 2 minutes in 1×10^{-4} M KMnO_4 . When eggs were immersed for longer durations or in higher concentrations than the above, most of them were swollen or cytolized. By the treatment with KMnO_4 (10^{-5} M) in a Ca-free condition, in spite of the fact that swelling of the egg or cytolysis was induced by a rather brief treatment, no cortical change was brought about. However, when the eggs thus treated with Ca-free KMnO_4 -sea water were transferred to normal sea water, the cortical change was immediately brought about, the jelly being extruded and subsequently the germinal vesicle broke down. When eggs were inseminated in Ca-free KMnO_4 -sea water and transferred immediately into normal sea water, the cortical change took place, but the eggs failed to develop beyond the breakdown of the germinal vesicle or the extrusion of the polar body. On the other hand, when eggs were inseminated

with normal or KMnO_4 (10^{-6} M)-treated spermatozoa in Ca-free sea water without KMnO_4 , they develop in normal way after they were transferred to normal sea water. This means that the treatment with dilute KMnO_4 for a short time is harmless on the spermatozoa and that the sperm-entrance into the egg is inhibited by some invisible change which has been induced on the egg surface by the treatment with Ca-free KMnO_4 -sea water. These results are shown in Table 1.

Table 1. Effects of KMnO_4 (10^{-6} M) on the unfertilized eggs of *Nereis japonica* (treatment for 10 minutes)

Treatments	KMnO_4 + sea water	KMnO_4 + Ca-free sea water	KMnO_4 + Ca-free sea water	KMnO_4 + Ca-free sea water ↓ Insemina- tion (30 seconds)	Ca-free sea water ↓ Insemina- tion (30 seconds)	Normal sea water ↓ Insemina- tion with KMnO_4 -pre- treated sperm
	Normal sea water	Normal sea water	Ca-free sea water	Normal sea water	Normal sea water	Normal sea water
Results	Breakdown of germinal vesicle	Breakdown of germinal vesicle	No change	Breakdown of germinal vesicle	Swimming larva	Swimming larva

Some other oxidizing reagents such as K_2CrO_4 , $\text{K}_2\text{Cr}_2\text{O}_7$, K-ferricyanide, K-ferrocyanide and NaClO_4 were also tested. The results are summarized in Table 2. K-ferricyanide and K-ferrocyanide were both effective. For example, eggs were activated in 0.01–0.001 M ferricyanide solution by 2 minutes' treatment, and even in a solution of 5×10^{-4} M, a long treatment achieved a weak activation. When K-ferrocyanide was used, a concentration higher than 0.01 M was required for activation. In a Ca-free condition, the effects of ferri- and ferrocyanide were quite similar to those of KMnO_4 in that, although some invisible reactions were supposed to have been evoked to block the sperm-entry, no visible change in the cortical layer took place.

The effects of K_2CrO_4 were found to be somewhat different from

Table 2. Effects of various oxidizing reagents on the unfertilized eggs (*N. japonica*)

Reagents	Concentration	Extrusion of jelly	Effects in Ca-free condition	Fertilizability after treatment
NaIO ₄	10 ⁻² M	—	—	+
KMnO ₄	10 ⁻⁶ M	+	(+)*	—
K ₂ CrO ₄	2 × 10 ⁻² M	+	—	—
K ₂ Cr ₂ O ₇	10 ⁻¹ M	—	—	+
K-Fericyanide	10 ⁻³ M	+	(+)	—
K-Ferrocyanide	10 ⁻² M	+	(+)	—
NaClO ₄	10 ⁻¹ M	—	—	+

* (+) means invisible effects, which become visible when removed to normal sea water.

the above reagents. It was effective at the concentration of 0.02 M, but not at 0.01 M. In a Ca-free condition, K₂CrO₄ produced no effect.

Neither K₂Cr₂O₇ nor NaClO₄ exerted any distinct morphological influence on eggs, except for a slight thickening of the surface membrane of the egg. The eggs treated with these reagents were fertilizable after they were returned to normal sea water, even when the treatment had been given for more than 30 minutes in 0.1 M solution.

There remains a question if the sperm-block of the egg induced by KMnO₄ and ferri- or ferrocyanide in a Ca-free condition is related to an oxidative reaction, since some reducing agents such as Na₂S₂O₃ and Na-thioglycolate did not cancel the effects of these oxidizing agents. When applied singly, these reducing agents had no effect on eggs, unfertilized and fertilized, even at the concentrations of 2–4 × 10⁻² M.

II. Effects of salts, non-electrolytes and detergents

Several salts such as NaCl, KCl, CaCl₂ and Na-oxalate, were tested, and only KCl was found to be effective for activation of the egg. When KCl solution, isotonic to 75% sea water, was added to 75% sea water in the ratio of 1 to 5, unfertilized eggs were activated in it. At lower concentrations of KCl, however, no activation occurred.

It has been known that urea is a strong activating agent for

sea urchin eggs (MOTOMURA 1934). In *Nereis* eggs, however, urea was found to be ineffective. Eggs were swollen and cytolized after 1 minute's treatment with 1 M solution. When the treatment was limited to 30 seconds, some of the eggs showed cytolysis, while the rest showed no visible change and were fertilizable if returned to normal sea water. The cytolysis induced by the urea treatment might be due to the absence of electrolytes, since 1 M glucose solution also induced cytolysis by 5 minutes' treatment.

Table 3. Effects of various reagents on the unfertilized eggs (*N. japonica*)

Reagents	Results
5/9 M KCl+sea water (in the ratio of 1:5)	Activation
Monogen 0.05%	Partial cytolysis after 1 minutes' treatment
Monogen 0.01%	Activation after 10 minutes' treatment
Digitonin 0.001%	Activation after 10 minutes' treatment
Na-cholate 0.05%	No effect, fertilizable in this solution
1 M Urea	Cytolysis after 1 minute's treatment
1 M Glucose	Cytolysis after 1 minute's treatment

Some detergents, such as digitonin, "Monogen" and Na-cholate were further tested. It was found that 0.05% Na-cholate was ineffective and the eggs were fertilizable even in this solution. On the other hand, 0.01% Monogen and 0.001% digitonin activated eggs by 10 minutes' treatment. 0.1% Monogen and 0.01% digitonin impaired an egg partially and subsequently caused its cytolysis. Such a partially impaired egg extruded the jelly layer, when it was inseminated. In a Ca-free condition, detergents induced neither the cortical reaction nor the sperm block, in which the action of detergents differed from that of KMnO_4 .

III. Effects of trypsin on the cortical reaction

The effects of trypsin on the surface of the sea urchin egg have been investigated by several workers (WICKLUND 1949, RUNNSTRÖM and WICKLUND 1949, BOHUS JENSEN 1953, HAGSTRÖM and HAGSTRÖM 1954, MOORE 1950). It has been known that formation of the fertilization membrane is inhibited when unfertilized eggs are treated with trypsin. In the present study, trypsin effect was tested on the

Table 4. Effects of digitonin on the unfertilized eggs (*N. japonica*)

Treatments	0.001% Digitonin sea water for 10 min. ↓ Normal sea water	0.001% Digitonin Ca-free sea water for 10 min. ↓ Ca-free sea water	0.001% Digitonin Ca-free sea water for 10 min. ↓ Normal sea water	0.001% Digitonin Ca-free sea water for 10 min. ↓ Normal sea water ↓ Insemina- tion	0.01% Digitonin sea water for 1 min. ↓ Normal sea water	0.01% Digitonin sea water for 1 min. ↓ Normal sea water ↓ Insemina- tion
	Results	Results	Results	Results	Results	Results
	Formation of 1st polar body	No change, some are cytolized	No change, some are cytolized	Normal fertiliza- tion	Partial cytolysis	Extrusion of jelly

egg of *Nereis japonica*. Trypsin powder (Bayer) was dissolved to 0.05% in sea water, or 10,000 H.U. of crystalline trypsin was dissolved in 100 cc. of sea water. The effects of trypsin on *Nereis* eggs were somewhat different from those on sea urchin eggs. In the former, the cortical layer broke down at fertilization and at activation by means of KMnO_4 , although a part of the cortex sometimes remained unchanged. However, the jelly layer was not extruded after the cortical reaction. When the treatment with trypsin was prolonged over 5 minutes, the egg membrane became slightly

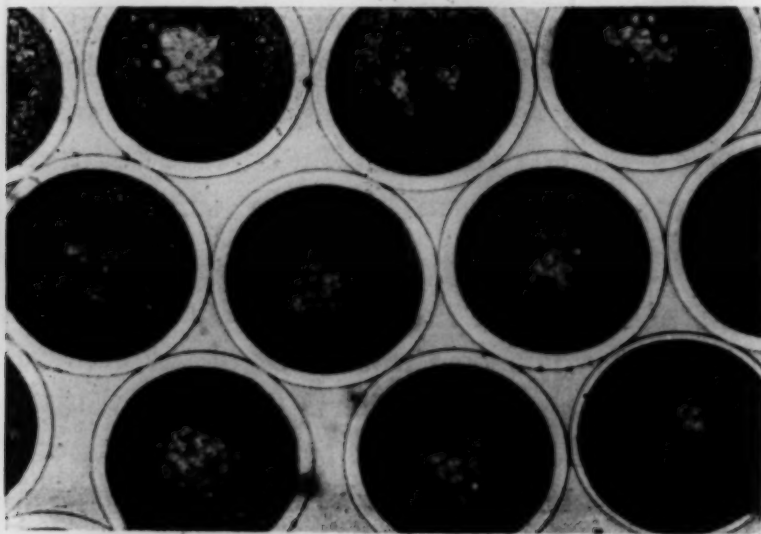


Fig. 2. Eggs inseminated after trypsin treatment, showing no jelly extrusion. ($\times ca. 140$)

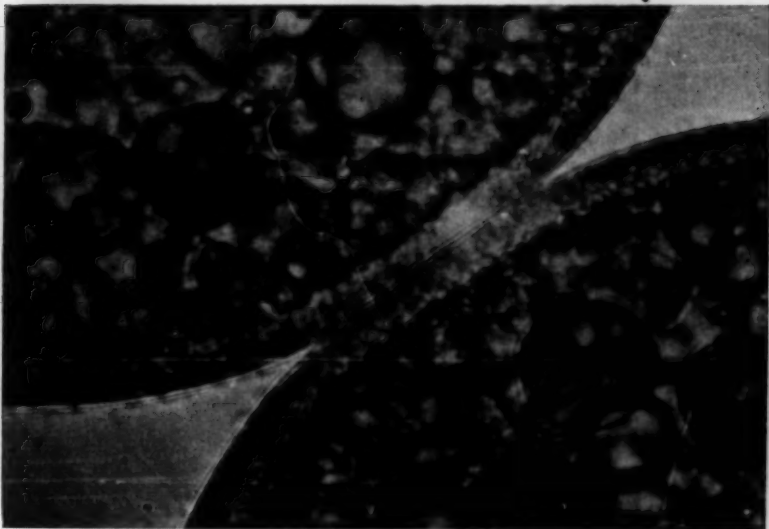


Fig. 3. Surface of unfertilized eggs. ($\times ca. 750$)

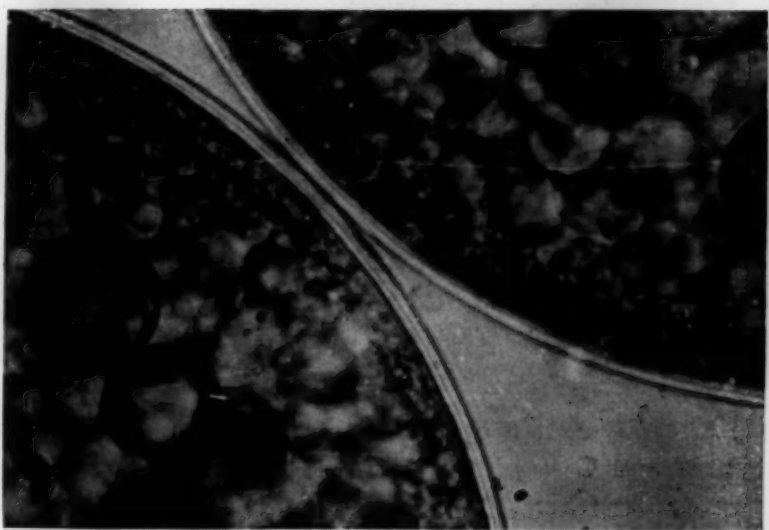


Fig. 4. Surface of unfertilized eggs after trypsin.
($\times ca. 750$)

thickened or lifted. The length of time of the trypsin treatment necessary to inhibit the extrusion of jelly was very short. Insemination just after the trypsin treatment could not induce jelly extrusion, even if the eggs were removed to normal sea water within 20 seconds. The effect was the same when the trypsin treatment and insemination had been started at the same time. In this case, it might be considered that trypsin had some effect also on the spermatozoa. This possibility was, however, denied, since the process of fertilization was quite normal when the normal egg was inseminated with the spermatozoa pretreated with trypsin for 5 minutes. It may

be said, therefore, that the effect of trypsin was limited to the egg.

A question arose whether trypsin affects eggs after fertilization. It was found that no jelly layer was formed when eggs were immersed in the solution within 1 minute after insemination, whereas in normal sea water it was formed between 1 and 2 minutes after insemination. In general, protein and mucoprotein conjugate with Ca-ions, and the absence of Ca-ions prevents the viscosity rise. It is possible either the jelly substance is not formed or it is softened and digested with trypsin in Ca-free condition. In the next experiments, effects of trypsin on inseminated eggs were studied in Ca-free condition.

Eggs were treated with Ca-free sea water for 5 minutes at 30 seconds to 3 minutes after insemination, and then transferred into the trypsin solution. 20 minutes after insemination, the eggs were examined. The jelly extrusion was found in the eggs which were treated 1 minute after insemination. In these eggs the perivitelline space was slightly enlarged. When the treatment was carried out at 30 seconds after insemination, the jelly layer was not formed. On the other hand, the jelly layer was formed even after transference to Ca-free sea water at 30 seconds after insemination so far as they were not treated with trypsin (Table 5), which means

Table 5. Jelly-extrusion of fertilized eggs pretreated with trypsin and Ca-free sea water as judged after 20 min.

Treatment	With trypsin*	With Ca-free sea water	With trypsin* after 5 min. treatment with Ca-free sea water
5 min. before insemination	—	+	—
1 min. "	—	+	—
At the time of insemination	—	—	—
30 sec. after insemination	—	+	—
1 min. "	—	+	+
2 min. "	+	+	+
3 min. "	+	+	+
5 min. "	+	+	+

* The concentration of trypsin is described in the text.

the initial step of fertilization reaction was completed within 30 seconds after insemination. The formation or hardening of the jelly layer was completed even in Ca-free sea water, although the process was slightly delayed. From the above it can be said that if the jelly layer was once formed, it was not digested by trypsin. Stating it otherwise, trypsin affected both unfertilized and fertilized eggs, so far as it was before the extrusion of the jelly layer. Since KMnO_4 failed to induced the formation of the jelly layer in trypsin-treated eggs, activation with KMnO_4 was quite similar to fertilization in these points.

DISCUSSION

RUNNSTRÖM and KRISZAT (1950) and KRISZAT and RUNNSTRÖM (1952) reported on activation of the sea urchin egg with periodate, and suggested that there are some relations between the oxidation of polysaccharide and activation or fertilization. Similar results were obtained in *Macra* eggs by SAWADA (1954, 1955). MOTOMURA (1954) also reported on the activation of *Caecella* eggs by KMnO_4 and suggested that the oxidation in the surface of eggs was related to activation. RUNNSTRÖM (1954) stated that the effect of periodate was found to be unique when compared with H_2O_2 , K-ferricyanide, iodate, iodine and iodosobenzoate. He reported also on the block of fertilization in the presence of porphyrin and porphyrinid (1957). In the present experiments, however, the author has found more complicated relations in *Nereis* eggs. KMnO_4 and K_2CrO_4 were effective for activation, while NaIO_4 , NaClO_4 , and $\text{K}_2\text{Cr}_2\text{O}_7$ were not. It is generally considered that the oxidizing power of KMnO_4 and K_2CrO_4 is stronger than that of NaIO_4 , NaClO_4 , and $\text{K}_2\text{Cr}_2\text{O}_7$. $\text{K}_3[\text{Fe}(\text{CN})_6]$ may be an oxidizing reagent, while $\text{K}_4[\text{Fe}(\text{CN})_6]$ is not; nevertheless both of them showed similar effects. Therefore, activation with these reagents is not necessarily the oxidation alone.

Some invisible changes seemed to have taken place in the surface of the eggs which had been treated with KMnO_4 and $\text{K}_3[\text{Fe}(\text{CN})_6]$ in a Ca-free condition, since the penetration of spermatozoa into the eggs was prevented after the treatment. A hasty conclusion must be avoided as to the question whether these invisible changes are of the same nature as that of the fertilization-wave in *Oryzias* eggs

(YAMAMOTO 1944) and sea urchin eggs (SUGIYAMA 1956).

Extrusion of the jelly layer in *Nereis* eggs may be comparable to the formation of the fertilization membrane in sea urchin eggs. The latter is inhibited by the pretreatment with trypsin as a result of digestion of the vitellin membrane (WICKLUND 1949, RUNNSTRÖM and WICKLUND 1949, etc.). In the present study, a comparable effect of trypsin was found in *Nereis* eggs. The formation of the jelly layer was inhibited by trypsin, when inseminated eggs were treated before its appearance. Since no jelly layer was formed in trypsin-treated eggs even when cortical granules broke down completely, it is concluded that the effect of trypsin on the jelly formation is not the result of inhibition of breakdown of the cortical granules. Once extruded jelly cannot be digested by trypsin. These facts mean that only the precursor of jelly substance can be digested by trypsin. The author performed the preliminary chemical analysis of jelly substance, and detected rather strong reactions of some amino-acids (aspartic acid, glutamic acid, serine, glycine and alanin) and unknown sugar in the hydrolysate of jelly substance by paper-chromatography. It may be said that jelly substance becomes indigestible after extrusion as the result of denaturation of protein moiety.

SUMMARY

1. KMnO_4 , K_2CrO_4 , K-ferricyanide and K-ferrocyanide are effective for activation of *Nereis* eggs, while NaCl , $\text{K}_2\text{Cr}_2\text{O}_7$ and NaIO_4 are not.
2. In Ca-free solution, KMnO_4 , K-ferricyanide and ferrocyanide produce an invisible effect on the egg surface, as the result of which the entrance of spermatozoa is inhibited. This effect cannot be removed by reducing reagents such as thioglycolate and $\text{Na}_2\text{S}_2\text{O}_3$.
3. Detergents such as digitonin and Monogen activate the egg at appropriate concentrations, but they do not evoke the invisible reaction in Ca-free sea water.
4. Urea induces swelling and cytolysis of the eggs, but it is ineffective for activation.
5. If eggs are treated with trypsin before insemination or before the appearance of the jelly layer after fertilization, extrusion of

jelly is inhibited, in spite of the breakdown of the cortical granules. This indicates that the precursor of the jelly layer is digested by trypsin and that the effect of trypsin is not due to inhibition of breakdown of the cortical granules.

6. The cortical layer contains the precursor of the jelly layer which is composed of protein and acid-mucopolysaccharide. The protein moiety is digested by trypsin only before the formation of the jelly layer.

The author wishes to express his gratitude to Prof. K. OKADA and Dr. Y. KAGAWA at Tokushima University and Prof. H. KINOSHITA at Tokyo University for their kind support and advice in carrying out the study. Author's gratitude is also given to Dr. SUGIYAMA of Nagoya University for revising the manuscript.

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STUDIES OF THE METAMORPHOSIS IN INSECTS

V. FACTORS CONTROLLING THE LARVAL PERIOD OF THE SQUASH FLY, *ZEUGODUCUS* *DEPRESSUS* SHIRAKI

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INTRODUCTION

The larva of the squash fly, *Zeugoducus depressus* SHIRAKI (Diptera) is parasitic on the squash. This species is distributed in the mountainous zones of several prefectures in Japan, such as Iwate, Gifu, Yamanashi, Toyama, Shiga and Nagano. The adult fly lays eggs into the unripe fruit of the host plant from the end of July to the end of September in Nagano Prefecture. The larvae hatch in several days and grow inside of the fruit. They stay in the cavity of the fruit without undergoing the pupation as long as the fruit remains on the vine. In the middle of December, when the mean atmospheric temperature is about 2°C and the fruit rots off on the ground, the larvae leave the fruit and go into the ground, where they pupate within 5-8 days. The length of the larval period is about 90-130 days under the natural condition.

When the eggs are taken out of the host fruit larvae hatch outside and they pupate within 26-32 days after oviposition (TAKAMATSU, 1952).

The shortening of the larval period, *i.e.* the acceleration of pupation, in the air suggests that the length of larval period is controlled by the internal conditions of the fruit-cavity, such as temperature, humidity, light and composition of the gas.

The present paper deals with limiting factors controlling the larval period. From studies on the constitution of the population within the fruits kept for various lengths of time, the analysis of the gas contained in the cavity of the fruit, and the rate of pupation of larva under experimental gaseous environment, it is revealed that the composition of gases in the cavity plays the decisive rôle

for the length of larval period.

The writer wishes to express his sincere thanks to Profs. TUNEO YAMADA and TAKESI MORI for their valuable criticisms and advices. Special thanks are also due to Mr. SADA O MATUO of the Institute of Earth Science, Faculty of Science, for gas analysis by the mass-spectrography and Mr. CHIAKI ITO of Shinshû University for supplying the materials.

MATERIAL AND METHOD

1. Experimental materials.

The materials used were larvae of *Zeugoducus depressus* SHIRAKI taken out of the squash. They could be cultured on the *Drosophila* food in good condition.

Squashes were collected in the Ina district of Nagano Prefecture on 7th September 1959 and were preserved in a dark room or in a dark incubator. The temperature and the duration of preservation are shown in Fig. 1.

NO. of sample	Period and thermal condition of preservation					
	10 days	30	70	100	133	206
NO.1-6	25° (20-30°)					
NO.7	22° (13-30°)					
NO.8,9			19° (13-25°)	25°		
NO.10,11				6.5° (0-13°)		
NO.12,13					25°	
NO.14,15				6.5° (0-13°)		5° (0-10°)

Fig. 1. Period and thermal condition of preservation. Numbers drawn on every line designate the temperature.

2. Preparation of the gas mixture.

As the gaseous environment for experimental purposes, 6% carbon dioxide and 16% oxygen were mixed with 78% nitrogen under one atmospheric pressure. Composition of the gas mixture is almost the same as that of the gas contained in the cavity of fresh fruits.

3. Exposure of the larvae to the gas mixture.

A glass bottle in which larvae were kept with the food was set

in a glass cylinder of about 3,600 cc. capacity. The technique for introducing the gas into the cylinder has been shown in previous papers (TAKAOKA, 1959, 1960).

4. Analysis of the gas in the cavity of the fruit.

The gas was collected in saturated NaCl or in 1/10 N H₂SO₄ solution with a syringe and was introduced into a gas collector. Analysis was carried out by the mass-spectrography or by the use of a gas analysis apparatus ("Roken"-type).

RESULTS OF OBSERVATIONS AND EXPERIMENTS

1. Composition of the gas in the cavity of fruits.

The gases contained in the cavities both of fresh fruits and of fruits which had been preserved for 206 days were analysed. The analyses were made in the former cases by the mass-spectrography, and in the latter cases with the gas analysis apparatus.

The results of the analyses are summarised in Table 1 and are graphed in Fig. 2.

Table 1. Compositions of gas contained in the cavities of fresh and preserved squashes

Sample			Constituent gas and volume fraction %				
			O ₂	CO ₂	O ₂ +CO ₂	N ₂	Ar
Air			20.95	0.03	20.98	78.09	0.93
Fresh squash	Non-infected	No. 1	18.12	3.25	21.37	78.72	0.91
		No. 2	15.93	5.46	21.39	77.69	0.94
		No. 3	17.47	3.98	21.45	79.65	0.89
		Mean	17.17	4.23	21.40	78.69	0.91
	Infected		16.02	6.00	22.02	77.04	0.93
Squash preserved for 206 days	Non-infected	No. 16	20.00	1.40	21.40	78.60	—
		No. 17	19.40	1.42	20.82	79.18	—
		Mean	19.70	1.41	21.11	78.89	—
	Infected	No. 15	20.10	0.80	20.90	79.10	—
		No. 12+	19.60	1.25	20.85	79.15	—
		No. 13#	14.78	7.20	21.98	78.02	—

+: Preserved at 25°C during the last 136 days in the course of preservation for 206 days.

#: The endocarp tissue was spoilt during preservation.

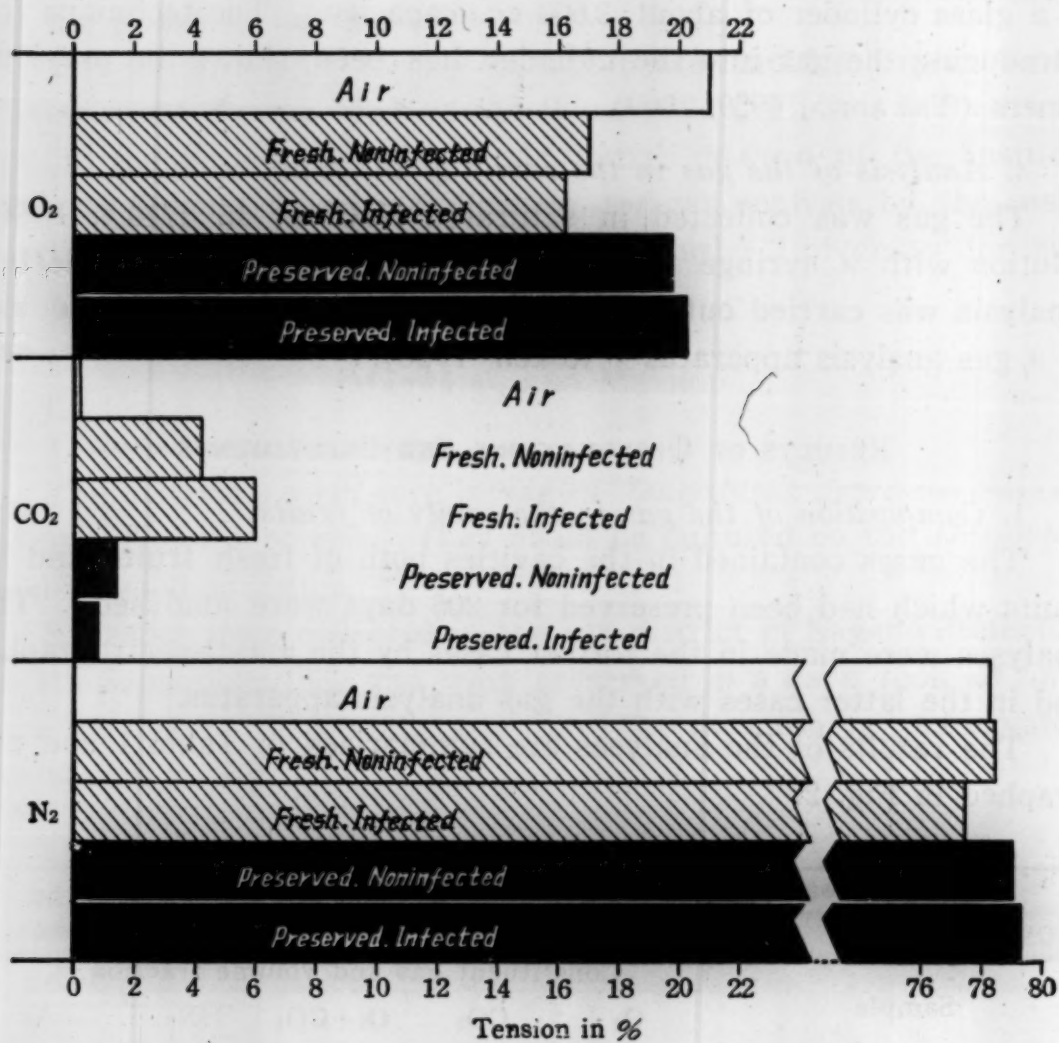


Fig. 2. Comparison of composition of the gas between the fresh and the preserved squash.

The data show that in the cavity of fresh fruits carbon dioxide tension was about 4% in the case of non-infected fruit and 6% in the case of infected fruit, Oxygen tension was about 16-17% in both of the cases and not so much less than that in the air.

In fruits preserved for 206 days, carbon dioxide tension decreased significantly (about 0.8% in infected fruits, 1.4% in non-infected fruits), whereas the oxygen tension increased slightly to the level in the air (19.7-20.1%).

As to nitrogen contents, there was no significant difference between the air and the gas contained in the cavity of both fresh and preserved fruits.

The present result of the gas analysis demonstrates that carbon

dioxide content in the fresh fruit is exceptionally high and estimated to reach about 200 times as much as that of the air, but it decreases during the course of preservation to a level of about 30-50 times. On the contrary, oxygen tension which is at first lower than in the air rather increases during the preservation for 206 days, so that as a whole, composition of the gas in the cavity of the fruit gradually approaches to that of the air during preservation.

2. Constitution of the population within the fruit.

Constitution of the population within the fruit was inspected on the 10th, 30th, 133rd and 206th day after preservation of fruits (Table 2).

As shown in Table 2, until the 30th day of preservation no pupa was observed in the fruit (Nos. 2 and 7).

Out of four fruits which were examined on the 133rd day, two fruits (Nos. 8 and 9) had been kept at 25°C., and the other two (Nos. 10 and 11) at 0-13°C. during the later half of preservation (63 days). In the former cases the pupation occurred in 46% of the population including 9 pupae just before emergence.

In the latter cases, when the fruit was not spoilt, pupation did not occur at all (No. 11). On the contrary, when the fruit was rotten by mold and the cavity was opened to the air during preservation a half of individuals (50%) underwent pupation (No. 10).

Examination on the 206th day of preservation was made on four fruits. One of them (No. 12) was kept at 25°C during the last 136 days. In the cavity of this fruit 3 (9.7%) imagoes and 17 (55%) pupae out of 31 individuals were found.

In the other two fruits (Nos. 14 and 15) which were preserved at 0-13°C. during the last 136 days the pupation occurred in 111 cases (60%) out of 186 individuals (Fig. 3).

When endocarp tissue of the fruit was spoilt and the CO₂-content became very high (7.2%), all larvae were perished within the fruit (No. 13).

The observations mentioned above suggest that CO₂ in a tension of 6% plays the most important rôle for the inhibition of pupation. The following series of experiments justify this suggestion.

3. Exposure of larvae to the air and to the gas mixture.

In this series larvae were exposed to the air and to a mixture

Table 2. Constitution of the population within the squash
The numbers in parentheses indicate the corresponding percentage

Sample	Duration of preservation (day)	Thermal condition during preservation	Constitution of population within the fruit				
			Imago	Pupa	Larva		Total
					Living	Perished	
No. 1, 3, 4, 5, 6	10	20-30°C. (for 10 days)	0	0	0	0	0
No. 2			0	0	26(100)	0	26
No. 7	30	20-30°C. (for 10 days) 30-13°C. (for 20 days)	0	0	62(100)	0	62
No. 8	133	20-30°C. (for 10 days)	0	25*	0	31	56
No. 9		30-13°C. (for 20 days)	} 55(46)	0	33	} 64(54)	} 119
		13-25°C. (for 40 days)					
		25°C. (for 63 days)					
No. 10 Δ		20-30°C. (for 10 days) 30-13°C. (for 20 days) 13-25°C. (for 40 days) 0-13°C. (for 63 days)	0	95(50)	10(5.2)	86(45)	191
No. 11			0	0	68(96)	3(4.0)	71
No. 12	206	20-30°C. (for 10 days)	3(9.7)	17(55)***	0	11(35)	31
No. 13 \circ		30-13°C. (for 20 days)	0	0	0	175(100)	175
		13-25°C. (for 40 days)					
		25°C. (for 136 days)					
No. 14		20-30°C. (for 10 days) 30-13°C. (for 20 days) 13-25°C. (for 40 days) 0-13°C. (for 63 days) 0-10°C. (for 73 days)	0	19	4	4	27
No. 15			0	92	33	34	159
				} 111(60)	} 37(20)	} 38(20)	} 186

Δ : The wall of the fruit was rotten by mold and the cavity was opened to the air during preservation (No. 10).

\circ : The endocarp of the fruit was spoilt during preservation (No. 13).

*: Including 7 pupae ready to emerge (No. 8).

**: Including 2 pupae ready to emerge (No. 9).

***: Including 1 pupa ready to emerge (No. 12).

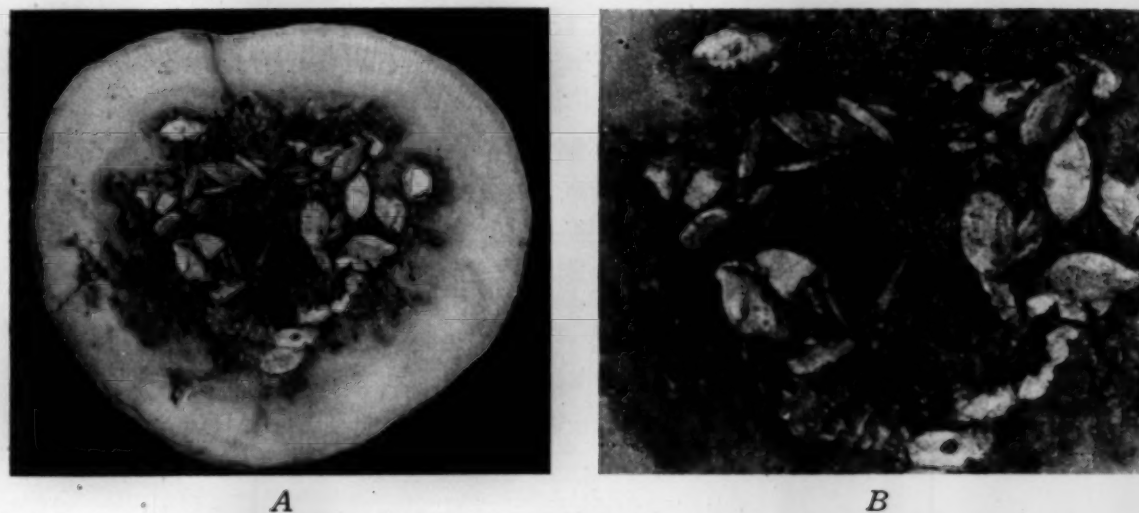


Fig. 3. A. Cross section of the squash preserved at 0-13°C. during the last 136 days in the course of preservation for 206 days (No. 15), showing pupae in the mesocarp and endocarp tissues. B. Enlarged endocarp of the fruit.

of 6% CO₂, 16% O₂ and 78% N₂ at various temperatures.

a) *Culture at 25°C. of the larvae, which were taken out after 30 days' preservation at 13-30°C. (No. 7), (Table 3).*

In the series with the air, 20 (67%) out of 30 larvae pupated within 18 days, but in the series with the gas mixture, larvae failed to pupate even after 26 days (Table 3). However, when one half of the survived larvae were put back in the air, they could pupate within 5 days but remainders left in the gas mixture failed to pupate and died after 20-26 days (Table 4).

Table 3. Pupation in the air and the gas mixture at 25°C. of larvae, which were taken out of the fruit kept at 13-30°C. for 30 days (No. 7)

The numbers in parentheses indicate the corresponding percentage

Days of exposure to gases	In the air (Total number of larvae: 30)			In the gas mixture (Total number of larvae: 32)		
	Pupated	Unpupated	Died	Pupated	Unpupated	Died
9	2(6.7)	28(93)	0	0	32(100)	0
15	4(13)	22(73)	4(13)	0	22(69)	10(31)
18	20(67)	0	10(33)	0	10(31)	22(69)
26	20(67)	0	10(33)	0	10(31)*	22(69)

* One half of these individuals were put back in the air and remainders were left in the gas mixture (Table 4).

Table 4. Pupation in the air and the gas mixture at 25°C. of larvae, which failed to pupate during the course of exposure to the gas mixture for 26 days at 25°C. (Table 3)

The numbers in parentheses indicate the corresponding percentage

Days of exposure to gases	In the air (Total number of larvae: 5)			In the gas mixture (Total number of larvae: 5)		
	Pupated	Unpupated	Died	Pupated	Unpupated	Died
5	5(100)	0	0	0	5(100)	0
10	5(100)	0	0	0	5(100)	0
20	5(100)	0	0	0	2(40)	3(60)
26	5(100)	0	0	0	0	5(100)

b) Culture at 25°C. and 0-10°C. of the larvae, which were taken out after 133 days' preservation (No. 11), (Table 5).

In the series with the air, 30 (94%) larvae pupate within 13 days at 25°C., and 19 (76%) larvae pupated between the 18th and 28th day at 0-10°C. In the series with the gas mixture, on the contrary, only 2 (18%) incomplete pupae out of 11 individuals were observed at 25°C. (Table 5).

Table 5. Pupation in the air and the gas mixture at 25°C. and 0-10°C. of larvae, which were taken out of the fruit preserved at 0-13°C. during the last 63 days in the course of preservation for 133 days (No. 11)

The numbers in parentheses indicate the corresponding percentage

Days of exposure to the gases	25°C.						0-10°C.		
	In the air (Total number of larvae: 32)			In the gas mixture (Total number of larvae: 11)			In the air (Total number of larvae: 25)		
	Pupa- ted	Unpupa- ted	Died	Pupa- ted	Unpupa- ted	Died	Pupa- ted	Unpupa- ted	Died
4	10(31)	22(69)	0	0	11(100)	0	0	25(100)	0
5	20(63)	12(38)	0	0	11(100)	0	0	25(100)	0
7	23(72)	7(22)	2(6.3)	0	11(100)	0	0	25(100)	0
13	30(94)	0	2(6.3)	2*(18)	9(82)	0	0	25(100)	0
18	30(94)	0	2(6.3)	2*(18)	7(64)	2(18)	3(12)	22(88)	0
28	30(94)	0	2(6.3)	2*(18)	7(64)	2(18)	19(76)	3(12)	3(12)

* Designates incomplete pupation.

The results show that the influence of different temperatures upon the pupation-rate is evident and that the pupation occurs better in the air than in the gas-mixture at the same temperature.

c) *Culture in the air at 25°C. of the larvae, which were taken out after 206 days' preservation (No. 15), (Table 6).*

As shown in Table 6, 17 (52%) larvae pupated within 10 days. On the 10th day of the experiment, 10 (30%) larvae were found alive, whereas 6 (18%) larvae dead.

When larvae of the squash fly are exposed to the gas mixture of almost the same composition as the gas in the fresh fruit, larvae cannot pupate. Even these larvae can pupate when they are put back in the air. Further, larvae can pupate in the fruit if its cavity is opened to the air during the course of preservation. On the contrary, if the endocarp tissue is spoilt and a large amount of carbon dioxide is accumulated in the cavity, all larvae are perished. All these facts clearly indicate that carbon dioxide plays the most important rôle for the inhibition of pupation.

Table 6. Pupation in the air at 25°C. of larvae, which were taken out of the fruit preserved at 0-13°C. during the last 136 days in the course of preservation for 206 days (No. 15)

The numbers in parentheses indicate the corresponding percentage

Days of exposure to the gas.	In the air at 25°C. (Total number of larvae: 33)		
	Pupated	Unpupated	Died
2	4(12)	29(88)	0
4	8(24)	25(76)	0
5	14(42)	17(52)	2(6.0)
10	17(52)	10(30)	6(18)

DISCUSSION

A comparable phenomenon to the results of the present experiments that pupation is inhibited by carbon dioxide has been observed also in *Drosophila*. In the mature larva of *Drosophila*, pupation is inhibited by concentration of carbon dioxide higher than 30-40% (TAKAOKA, 1960), and when larvae are cultured from hatching in the gas mixture of 5% carbon dioxide with 21% oxygen at 25°C., larval period is prolonged in comparison with that in the air (TAKAOKA, unpublished).

Absence of light (darkness) and high humidity within fruits

seem to be negligible as inhibiting factors of pupation at least under the present experimental condition, because larvae can pupate in the dark and moist cavity or succulent mesocarp tissue of the fruit, when it is preserved for long period or the cavity of the fruit is opened to the air during preservation.

There is no doubt that the thermal condition is one of the important factors controlling larval development. However at different temperatures (0-10° and 25°C.) since the time required before pupation varied considerably which, in turn, resulted in wide varieties of population constitution (cf. Table 5 and Table 2; Nos. 8, 9 and No. 11, No. 12 and Nos. 14, 15). It seems, therefore, that the thermal condition though it is controlling factor for the development and metamorphosis in general, it may not be specific factor for initiation of the pupation.

In the light of the results above discussed, it can be concluded that the suppressing factor of pupation within the host fruit is high level of carbon dioxide in the cavity of the fruit.

Therefore, the life history of the squash fly can be interpreted as follows. The eggs are laid in the unripe squash in summer in which the larvae of the fly hatch and grow. They do not pupate while the content of carbon dioxide in the fruit-cavity is high. If the host fruit happens to rot, larvae pupate in a short time by coming into the air earlier than usual. This is also true in experimental exposure of the cavity to the air under adequate temperature (Table 2. No. 10). In other words, adequate temperature and decrease in the CO₂-content enable the larvae to pupate.

If the temperature in the fields falls, the larval period is prolonged and the pupation does not occur before the time when the fruits are rotten by cold in winter and the larvae can then leave the fruit for pupation.

SUMMARY

The squash fly, *Zeugodacus depressus* SHIRAKI deposits eggs in the squash and larvae live in the fruit about 90-130 days before they leave it and migrate for pupation under ground. When the egg is removed from the host fruit and cultured in the air, the larval period becomes much shorter. The results of the efforts to

find the controlling factors of the larval period were as follows.

1) Carbon dioxide tension in the cavity of the fresh fruit is exceptionally high (about 4.2-6.0%), which decreases during preservation. After 206 days, the carbon dioxide content becomes about 0.8-1.4%. On the contrary, since the O₂-tension in the cavity remains 19.7-20.1%, the composition of the gas within the fruit approaches to that of the air during preservation.

2) In the gas mixture of 6% CO₂, 16% O₂ and 78% N₂, the pupation is reversibly inhibited.

3) When the cavity of the fruit is opened to the air larvae can pupate, whereas within the well preserved fruit, they fail to pupate under the same thermal condition.

4) The high content of carbon dioxide in the cavity affects the length of the larval period.

5) After 133-206 days of preservation, larvae can pupate and emerge inside of the host fruit as the result of decrease of carbon dioxide tension in the cavity.

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STUDIES ON THE TELOBLASTS IN THE DECAPOD EMBRYO

II. ORIGIN OF TELOBLASTS IN *PAGURUS SAMUELIS* (STIMPSON) AND *HEMIGRAPUS* *SANGUINEUS* (DE HAAN)

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INTRODUCTION

Last year, the author (1959) published a work on the teloblasts of a macruran, *Heptacarpus rectirostris* (STIMPSON) with particular reference to their origin, which had not been exactly known in any of the malacostracans with the teloblastic ring. In order to ascertain whether the fact found in the above named shrimp may or may not be available for other suborders of the Decapoda, she has made a similar study on an anomuran, *Pagurus samuelis* (STIMPSON) and a brachyuran, *Hemigrapsus sanguineus* (DE HAAN).

The presence of the teloblasts in the anomuran embryos was noticed by KRAINSKA (1936) in her embryological studies on *Eupagurus prideauxi* LEACH, though there were certain inexactness in her statement respecting their origin. In the brachyuran embryos, even their presence or absence has, thus far, not yet been confirmed. The present study has revealed that these crabs exhibit no essential difference from *Heptacarpus* with respect to the origin of the teloblasts except for some minor points. This appears to suggest us that the differentiation of the teloblasts may proceed in a more or less similar way throughout the entire group of the decapods. In the following pages, therefore, the description will chiefly be confined to pointing out the differences observable in *Pagurus* and *Hemigrapsus*.

The author wishes to express her sincere thanks to Prof. S. M. SHIINO of our Faculty for his kind guidance and for his valuable suggestions given to her during the whole course of the study. Her thanks are also due to

Dr. M. SUGIYAMA for his kindly offering every facility for collecting the material.

MATERIALS AND METHODS

The egg-bearing females of *Pagurus samuelis* (STIMPSON) were collected at Sugashima Island, Mie Prefecture in the Spring of 1959. Those of *Hemigrapsus sanguineus* (DE HAAN) were gathered on the seashore in Tsu, Mie Prefecture in the summer of the same year. The eggs, which were removed from about 150 females in both the crabs, were fixed with alcoholic BOUIN's solution. The methods of making a toto-preparation and serial sections were mentioned in the author's previous report and there may be no need of repetition.

OBSERVATION

Both in *Pagurus* and in *Hemigrapsus*, the ectoteloblasts are 19 in their total number and include the unpaired central teloblast. They are arranged on the superficial layer of thoracico-abdominal process, which they surround forming a complete ring. Eight mesoteloblasts lie just beneath them as in the case of *Heptacarpus* (Figs. 3-A-6, B-6, C-5). In denominating each of them the author follows what has been employed in the study of *Heptacarpus*. The differentiation of the teloblasts and the completion of their number take place during the stages from the formation of the germinal disc to the metanauplius as in *Heptacarpus*. In the present work, therefore, the author dares to make no mention of the way of subdividing the stages into minor ones in which the teloblasts differentiate.

Ectoteloblasts. In the stage of germinal disc formation, the ectoteloblasts of *Pagurus* and *Hemigrapsus* make their first appearance on both sides of the peri-blastoporal area as paired groups of four cells (Figs. 1-1 and 2-1). Their arrangement and morphological features in this stage are exactly the same as in *Heptacarpus*. They are denoted on each side by E1, II, III and IV from the inside to the outside, E1 representing the first of the appearing teloblasts and the others the mother cells of the second (E2) to the eighth teloblasts (E8) (Table 1). As in *Heptacarpus*, the first teloblastic division is undergone, earliest of all, by E1, and the division with which the mother cells yield additional teloblasts begins with II

Table 1. Differentiation of teloblasts in *Pagurus samuelis* (STIMPSON) and *Hemigrapsus sanguineus* (DE HAAN)

Ectoderm	E 0 { e 01 E 0	
	E 1 { e 11 E 1	
	II {	E 2 { e 21 E 2
		II 1 { E 3 { e 31 T 3 E 3
	III {	III 1 { E 4 { e 41 T 4 E 4
		E 5 { e 51 E 5
	IV {	(Pagurus) { E 6 { e 61 E 6
		IV 2 { E 7 E 8
		IV 1 { E 6 { e 61 E 6
		(Hemigrapsus) { E 7 { e 71 E 7
		T 5 { E 7 { E 7 E 8
	(Pagurus) E 9	
	(Hemigrapsus) E 9	
Mesoderm	M'I { M'I 1 { M 1 M 3 M 2	
	M 4	

(Figs. 1-2, 2-2 and Table 1). The series of mitoses which the mother cells II and III undergo gives the origin, exactly in the same way as in *Heptacarpus*, to the teloblasts from E2 to E5 and the telson ectoderm cells T3 and T4 (Figs. 1-2~5, 2-2~5 and Table 1). In other words, respectively with two divisions, the cell II is divided into E2, E3 and T3 and the cell III is into E4, E5 and T4.

In *Pagurus* and *Hemigrapsus*, the central teloblast (E0) becomes differentiated from one of the peri-blastoporal cells independently of other teloblasts when the cell III divides into III1 and E5 (Figs. 1-3 and 2-3). In *Heptacarpus*, it is rather difficult in this stage to discriminate the cell E0 from the other ordinary cells (cf. ŌISHI, 1959, p. 295, Fig. 1-6). Subsequent to the division of the cell III, the mother cell IV divides into larger and smaller cells, IV1 and T5 (Figs. 1-4 and 2-4).

As in *Heptacarpus*, the daughter cells resulting from the equal division of IV1 of *Hemigrapsus* are the teloblasts E6 and E7 (Fig. 2-6), both of which give birth to their first descendants in the next stage (Fig. 2-7). In *Pagurus*, on the other hand, daughter cells represent the teloblast E6 and the mother cell IV2 (Figs. 1-6~7 and 3-B-3), since, unlike E6 which successively enters the teloblastic division, IV2 is again divided into two teloblasts. The axis of the mitotic spindle of IV1 in *Pagurus* and *Hemigrapsus* is oriented toward the median line of the egg in contrast to what is found in *Heptacarpus*, where it is directed backward (Figs. 3-A-2, B-3, C-2). With those divisions, the teloblasts which have arranged themselves in a half ring gradually become to form a ring which is, for a time, open on the back.

The ninth teloblast E9 derives its origin on each side from one of the ordinary peri-anal cells as in *Heptacarpus*, but differentiating somewhat earlier. In *Hemigrapsus*, the differentiation takes place during the time when the division of the cell IV1 is proceeding and in *Pagurus* it does after the end of this division (Figs. 1-7, 2-7 and 3-A-5, B-4, C-2).

Subsequently to the completion of the teloblastic ring which is composed of 17 cells, the cells IV2 in *Pagurus* is equally divided into two cells, the teloblasts E7 and E8, both of which intrude between E6 and E9. E7 of *Hemigrapsus* gives forth E8 which becomes to lie just inside the E9. The differentiation of the ectoteloblasts

in both the species thus comes to an end and the teloblastic ring comprises the full number of 19 cells (Figs. 1-8, 2-8 and 3-B-6, C-5). In surface view, several of them which are located on the ventral side of the caudal papilla are recognizable with some difficulties owing to the folding of the papilla, which extends forward a little beyond the posterior margin of the mandible. In *Heptacarpus*, on the contrary, the named structure does not stretch forward in this stage so as to conceal the ventral members of the teloblasts from view.

A rapid development of the papilla is ascribable to the vigorous multiplication of the cells constituting the rudiments of maxillae

Explanation of Abbreviations in Figures 1-3

an 2: second antenna. as: anus. E0: central ectoteloblast. E1-E9: first to ninth ectoteloblasts. e01-e02: first to second descendants of E0. e11-e14: first to fourth descendants of E1. e11'-e11'': daughter cells of e11. e21-e23: first to third descendants of E2. e21'-e21'': daughter cells of e21. e31: first descendant of E3. e42-e43: second to third descendants of E4. e51: first descendant of E5. e61: first descendant of E6. e71: first descendant of E7. e81: first descendant of E8. M'I: mother cell of M3 and M'I1. M'I1: mother cell of M1 and M2. md: mandible. T0-T7: telson ectoderm cells which are in contact with the inner side of the teloblastic ring. x1-x5: maxillary ectoderm cells. x11-x12: daughter cells of x1. x11'-x11'': daughter cells of x11. x12'-x12'': daughter cells of x12. x21-x22: daughter cells of x2. x31-x32: daughter cells of x3. x41-x42: daughter cells of x4. x51-x52: daughter cells of x5. II: mother cell of E2 and II1. II1: mother cell of E3 and T3. III: mother cell of E5 and III1. III1: mother cell of E4 and T4. IV: mother cell of IV1 and T5. IV1: mother cell of E6 and E7 or IV2. IV2: mother cell of E7 and E8.

Fig. 1

Ventral plate of *Pagurus samuelis* (STIMPSON) in successive stages of its development, surface view. $\times 350$.

1. First appearance of ectoteloblasts.
2. Mother cell II in karyokinesis which gives rise to E2 and II1.
3. Division of III into III1 and E5.
4. Mother cell IV in karyokinesis which gives birth to IV1 and T5.
5. Half ring formed by 11 ectoteloblasts (E0 and E1-E5 of both sides).
6. Division of IV1 into E6 and IV2.
7. Provisional ring of 17 ectoteloblasts.
8. E7 and E8 produced by the division of IV2.

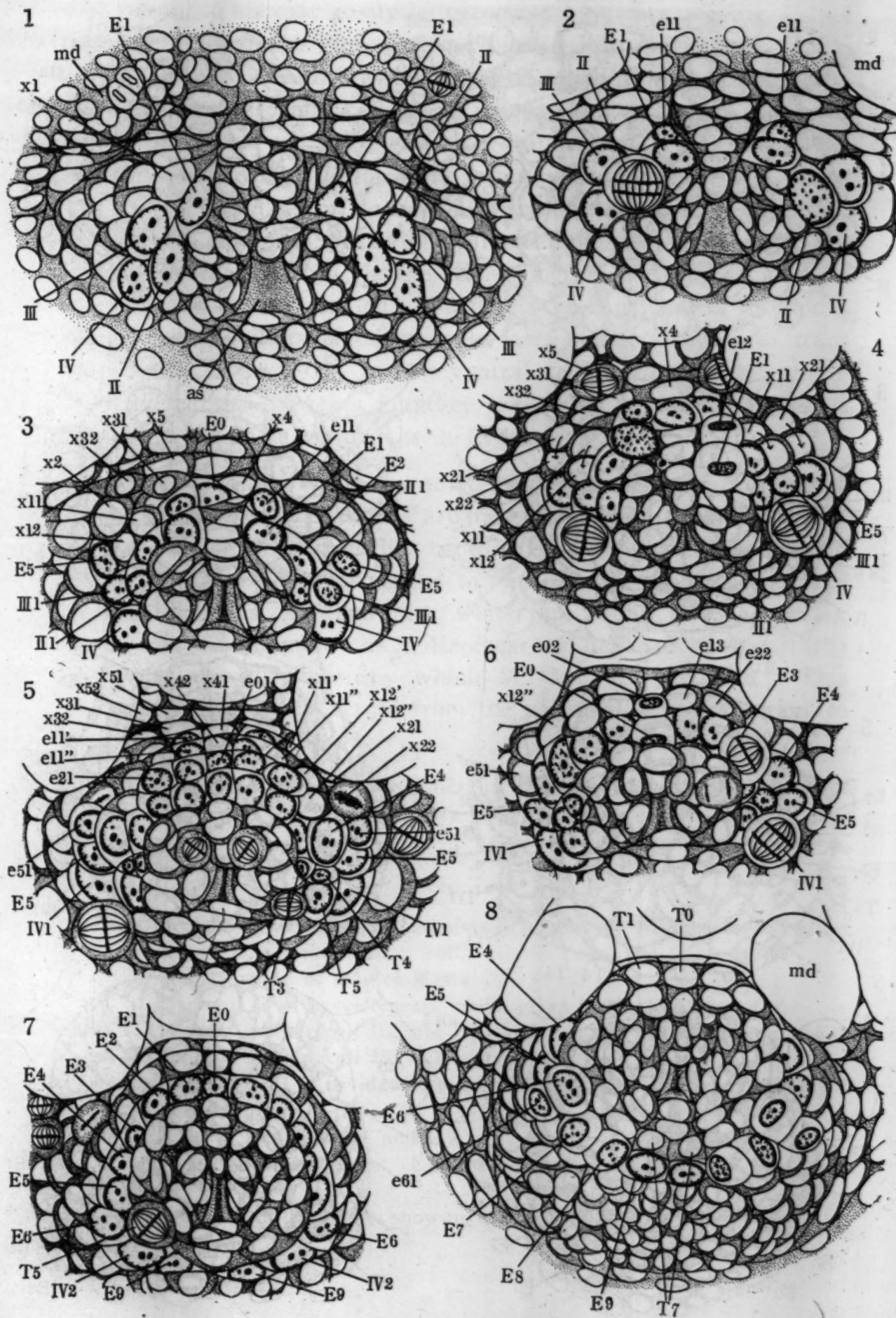


Fig. 1

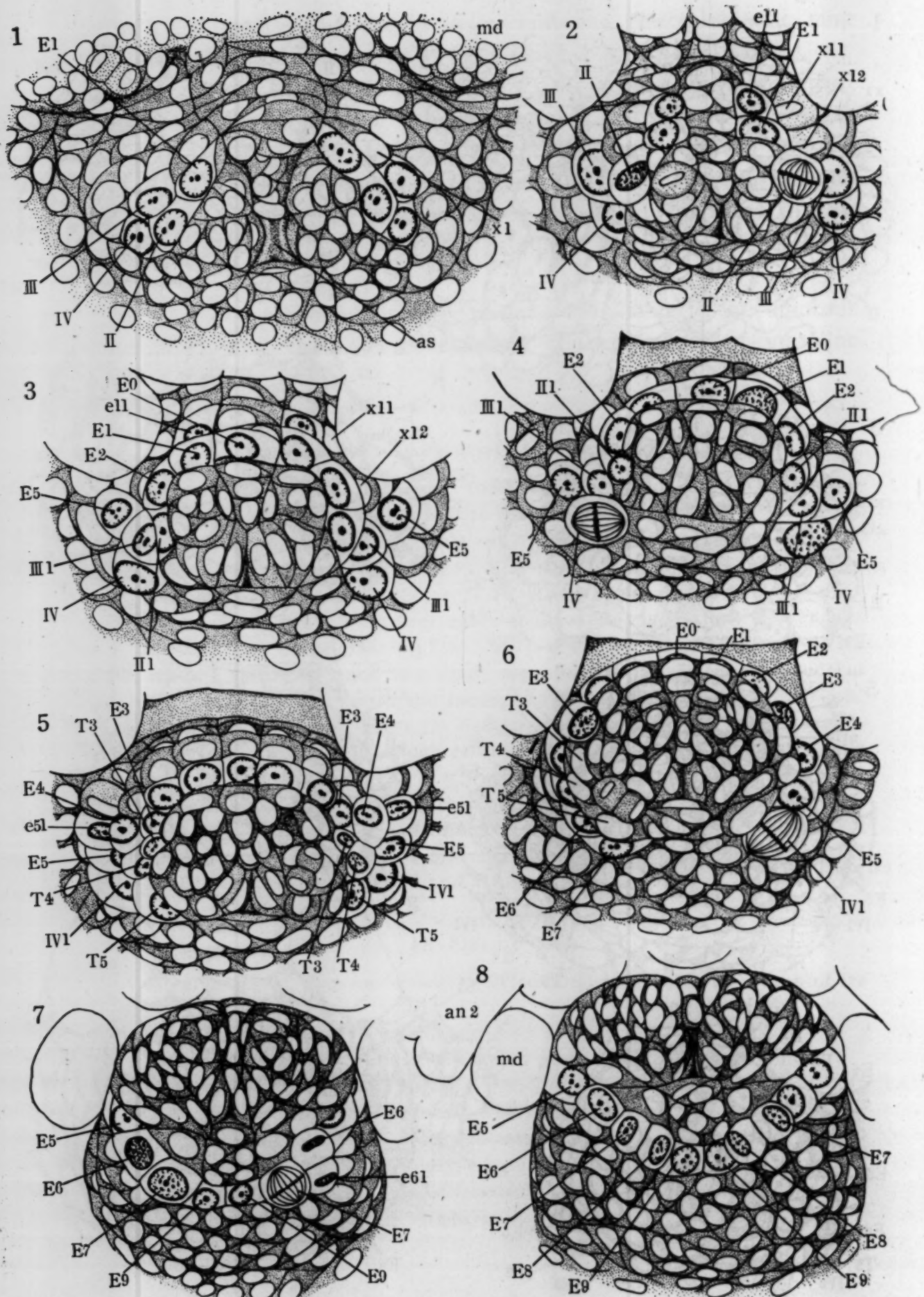


Fig. 2

and telson. This may easily be perceived by comparing Fig. 1-1~5 and Fig. 2-1~3 with Figs. 1-2, 3, 5, 6 and Figs. 2-9, 11, 13, 15 illustrated in the author's previous paper on *Heptacarpus*. For instance, the maxillary ectoderm cell x1 of *Hemigrapsus* undergoes a division when the mother cell II gives birth to E2 and II1, whereas the corresponding cell of *Heptacarpus* is not yet divided in this stage. The cells x3 and x5 of *Pagurus* prepare for the division when IV1 and T5 are produced. The above-named cells of *Heptacarpus* are divided for the first time in the succeeding stage, in which E3 and T3 differentiate, or later on. Owing to the rapidness in the multiplication of maxillary ectoderm cells, it is rather difficult to trace the process of formation of the ventral teloblastic descendants in *Pagurus* and *Hemigrapsus*, but they may, with all probabilities, be yielded in the same number as in *Heptacarpus*.

Mesoteloblasts. When the ectoteloblasts make their first appearance, two pairs of large cells are detectable within the mesodermal cell-complex which lies beneath the blastopore. Considering from their fates, they correspond to two pairs of the ancestral cells of mesoteloblasts, M'I and M4, which are found in *Heptacarpus*. Exactly in the same way as in *Heptacarpus*, one pair of them (M'I) undergoes two divisions, by which M1-M3 are produced. This takes place during the stages from the germinal disc formation to the prenauplius (Table 1).

Telson ectoderm. As mentioned in the previous work, the peri-

Fig. 2

Ventral plate of *Hemigrapsus sanguineus* (DE HAAN) in successive stages of its development, surface view. $\times 675$.

1. First appearance of ectoteloblasts.
2. Mother cell II in karyokinesis which gives birth to E2 and II1.
3. Division of mother cell III into III1 and E5, and differentiation of E0.
4. Division of mother cell IV which gives rise to IV1 and T5.
5. Mother cell III1 is divided into E4 and T4, and teloblast E5 gives off its first descendant.
6. Division of IV1 into E6 and E7.
7. Ring of 17 ectoteloblasts. E6 and E7 are emitting their first descendants.
8. Ring of 19 ectoteloblasts, showing E8 given off from E7.

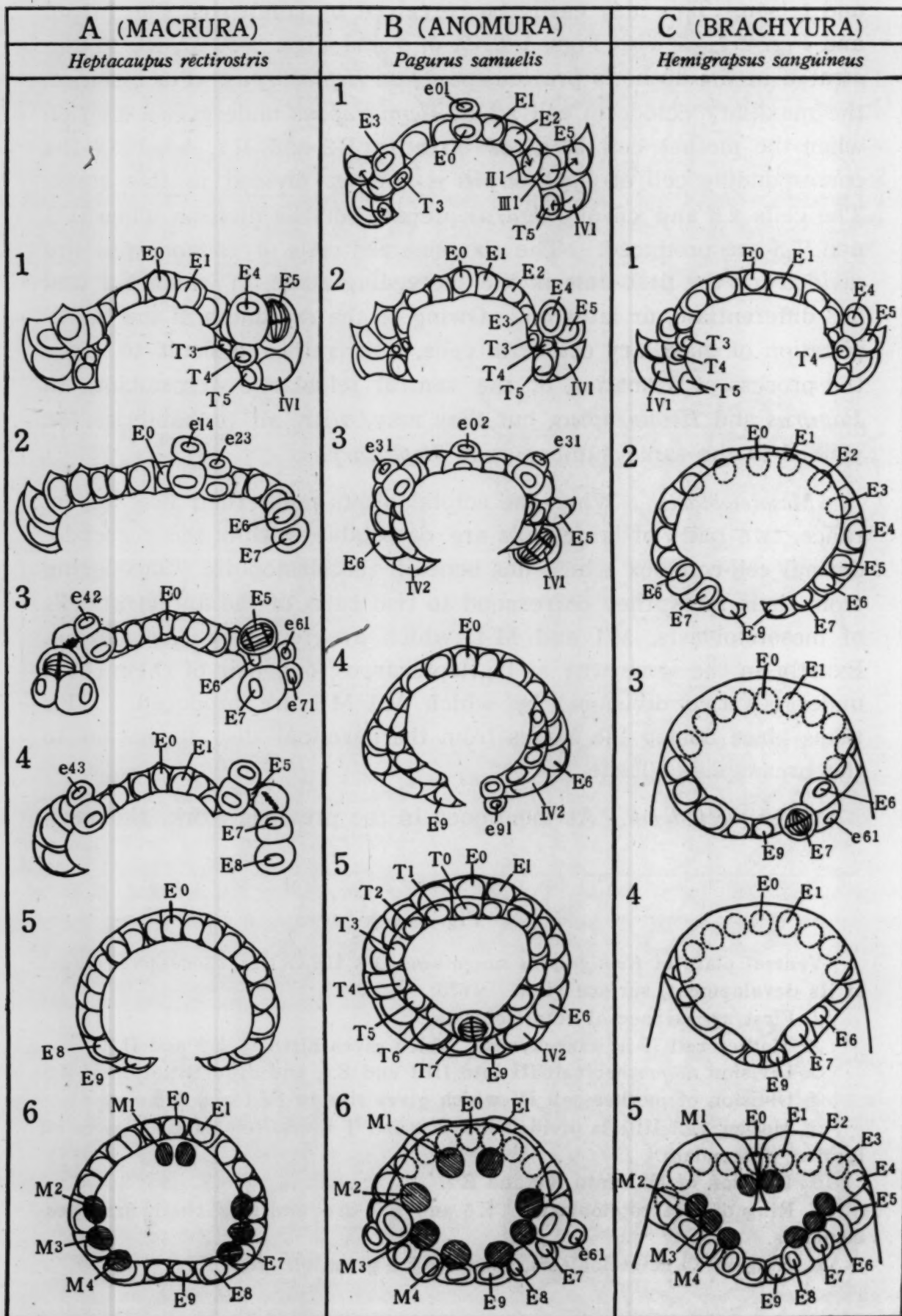


Fig. 3.

blastoporal area surrounded by the ectoteloblasts represents the first rudiment of the telson, the formation of which is in an intimate relation to the differentiation of ectoteloblasts. However the accurate number and origins of all the telson ectoderm cells along the inner side of the teloblastic ring cannot be ascertained on this occasion. They consist of 15 cells, of which the one lying on the mid-ventral line is denoted by T0 and the others by T1-T7 from the inside to the outside (Fig. 1-8). Although in *Heptacarpus* their origin has already been clarified for the greater part, that of T7 remains obscure. *Pagurus* is a material particularly suitable for tracing the origin of this cell. During or after the division of T5, by which T6 becomes differentiated, a single cell situated just behind the anus undergoes an equal division with its spindle orienting at right angles to the mid-dorsal line (Figs. 1-7, 8 and 3-B-5), and the daughter cells thus yielded become the pair of T7. In this way, of the 15 telson ectoderm cells seven (unpaired T0 and pairs of T1, T2 and T7) take their origin from the peri-blastoporal area and eight (pairs of T3-T6) arise from the ectoteloblasts.

CONSIDERATION

Pagurus samuelis (STIMPSON) and *Hemigrapsus sanguineus* (DE HAAN) are in accord with each other in that the ectoteloblasts of earlier stages are represented by two lateral groups, each consisting of four large cells (E1, II, III, and IV). Increase of the teloblasts in their number is effected by the division of the mother cells and by the differentiation of the cells located on the blastoporal area. The process of the formation of teloblastic ring observed in those decapods is again for a great measure coincident with what has already been described with respect to *Heptacarpus*. From this fact one will naturally reach the conclusion that a similar course is followed in other decapods.

KRAINSKA claims that the teloblasts of *Eupagurus prideauxi* LEACH

Fig. 3.

The differentiation of teloblasts and the changes in their arrangement from a half ring to a complete ring in macrura, anomura and brachyura.

are increased in their number not by the division of mother cells, but merely by the differentiation of ordinary blastoporal cells. In her Fig. D, P 1, xx 1, among the teloblasts, which do not contain the unpaired central one, there are two cells in karyokinesis lying just outside the cells which may correspond to the E 1 pair of *Pagurus* (cf. KRAINSKA, 1936, p. 558). The blastoporal area showing such a feature can be discovered in Fig. 1-2 of the present work. Those cells figured by KRAINSKA as dividing may be the mother cells II of *Pagurus* and others, which are in the course of division into the teloblast E 2 and the mother cell II 1. KRAINSKA, without elucidating the fate and behavior of those cells in question, arrived at an erroneous conclusion that all the teloblasts on the ventral side (six to seven cells on each side according to her) commence to give off their descendants as early as the stage in which only one pair of teloblasts (E 1) of *Pagurus* and others begin teloblastic division.

There is another mistake in her statement on the number of teloblasts. She says 17 of these form a ring in *Eupagurus*: As stated before, the ring of 17 cells is also found in *Pagurus* and *Hemigrapsus*, which is only a temporary formation observed prior to the perfection of the full number. When perfected, the number becomes 19 with the addition of a new pair (E 8) which is derived from IV 2 in *Pagurus*, or from E 7 in *Hemigrapsus*. The ring-formation preceding the attainment of the full number may be the result of the rapid growth of the caudal flexure in the region between the mandibles and the ventral plate. This may also be the case with *Eupagurus*. KRAINSKA's mistake appears to be due to the fact that she saw nothing but the transient ring. It is presumed that the ring of *Eupagurus* may also comprise 19 cells of the similar origin as that in *Pagurus*.

Even after the completion of their full number, the teloblasts of *Heptacarpus* remain for a while to form an incomplete ring open on the posterior side (Fig. 3-A-5). In contrast to the other suborders, the Macrura appears to be characterized by an earlier perfection in the number of teloblasts. In *Leander* (SOLLAUD, 1923) these cells are said to be 20 to 22 in number and in *Caridina* (NAIR, 1949) and *Palaemon* (AIYER, 1949) 21. The number surpassing 19 may probably be ascribed to an error for including a few non-teloblastic cells found between the ninth teloblasts of both sides. It is highly

probable that their total number is always 19 in the decapods and does not vary according to species.

In conclusion, *Pagurus* and *Hemigrapsus* differ from *Heptacarpus* only in forming a provisional ring of 17 ectoteloblasts, and the former is unlike the others in that the dorsal teloblasts E7 and E8 are derived from the mother cell IV 2. Such differences, however, are minor in importance as compared with such a great conforming as that the ectoteloblasts arising from the peri-blastoporal cells are confined to E0, E1 and E9 and the remainders E2-E8 are the derivatives of the mother cells II, III and IV.

SUMMARY

1) For determining the cell-lineage of teloblasts in the Anomura and Brachyura, *Pagurus samuelis* (STIMPSON) and *Hemigrapsus sanguineus* (DE HAAN) were respectively selected as material.

2) The ectoteloblasts in both the decapods are 19 in their full number.

3) They form a provisional ring of 17 cells and the members which differentiate last do not belong to the ninth pair, E9, but to the seventh and eighth, E7 and E8, in *Pagurus*, or E8 in *Hemigrapsus*. This difference is of a minor importance.

4) The mesoteloblasts are eight in number and they take their origin exactly in the same way as in *Heptacarpus rectirostris* (STIMPSON).

5) The telson ectoderm which is in direct contact with the ectoteloblastic ring on its inner side consists of 15 cells, which are partly derived from four pairs of daughter cells of ectoteloblasts.

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SKELETON FORMATION OF SEA URCHIN LARVAE

II. ORGANIC MATRIX OF THE SPICULE

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INTRODUCTION

Although several workers have reported that spicule formation in sea urchin larvae takes place in an organic mass surrounding the spicule, the presence of a skeletal matrix as understood in connection with shell formation in molluscs has not yet been generally accepted. In molluscs, it is now conceded that there are two distinct phases in the elaboration of the shell: (1) the formation of a protein membrane; and (2) the mineralization of this membrane which results in the production of the calcified exoskeleton (BEVELANDER and BENZER, 1948; BEVELANDER, 1952; HIRATA, 1953).

As will be reported in the present paper, there is no doubt that an organic matrix is formed preceding spicule formation in sea urchin larvae. To analyze the cause of the failure of persuasion in the past, an historical survey will first be presented, with particular emphasis on the structure in which the calcareous spicule is formed.

Historical survey

SELENKA (1879), who was the first to treat the problem of the mode of calcareous deposit in sea urchins (*Echinus miliaris*, *Toxopneustes brevispinosus*, *Strongylocentrotus lividus*, *Arbacia pustrosa* and *Echinocardium cordatum*), reported that a minute calcareous body began to be deposited as a skeletal rudiment in the interior of two primary mesenchyme cells located in bilaterally symmetrical positions in the gastrula. When the rudiment developed to acquire a triradiate form, the cell which formerly contained the calcareous granule moved toward one arm of the spicule, while two new primary mesenchyme cells came to the two remaining arms to supply them with calcareous salts. His description implicitly suggests that the spicule is intracellular at first, but becomes extracellular some time later, although he did not commit himself clearly on this point.

SEMON (1887) also observed a calcareous rudiment originating in the interior of a primary mesenchyme cell in the gastrula of *Strongylocentrotus lividus*. He further found that, when the rudiment developed to the triradiate form, the spicule was already outside the cell and was surrounded by a thin homogeneous membrane. SEMON, however, was not certain whether the spicule had come out of the cell or whether the cell itself had transformed into the investing membrane by losing its nucleus.

THÉEL (1892) studied both living and fixed larvae of *Echinocyamus pusillus*, and observed (1) primary mesenchyme cells containing one or more minute granules and (2) a calcareous tetrahedron within "clear pseudopodial plasm" which is formed by the fusion of the pseudopodia of at least three primary mesenchyme cells. He put the two separate observations together and conceived the process of calcification as follows. (a) The primary

mesenchyme cells must be saturated with calcareous matter, any surplus of which, if present, must be deposited as reserve granules. (b) For the formation of a spicule, the primary mesenchyme cells are first combined into a common clump by means of their pseudopodia; this serves as an organic substratum in which calcification takes place. (c) Calcareous salts are continuously carried to the clump in fluid form, and deposited there, while the reserve granules in the cells may be dissolved to supply material for further deposition on the spicule. He further inferred that (d) after the deposited calcareous matter has developed into a triradiate or more advanced form, the main cytoplasm of the primary mesenchyme cells may move along the arm of the spicule while new cells would enter into communication with the original "pseudopodial clump" which had meanwhile transformed into a thin investment surrounding the spicule. Thus, all the primary mesenchyme cells must eventually cooperate in forming the organic ground substance and in supplying calcareous salts. It is doubtful, however, whether he actually observed such an organic envelope on the spicules at various stages, since he illustrated the structure only around the rudimentary tetrahedron.

In sectioned gastrulae of *Echinus esculentus*, WOODLAND (1906) observed three states of skeletal development: (1) small calcareous granules within the primary mesenchyme cells, (2) young triradiate spicules with "faint streaks" projecting from the tips of the rays, (3) larger triradiate spicules growing within the "protoplasmic process" connecting the primary mesenchyme cells. If the streaks of (2) were traced away from the tips of the rays, they were found to be continuous with the "protoplasmic cord" on which the primary mesenchyme cells were strung. It is not clear whether or not he thought that the structures which he termed "faint streak", "protoplasmic cord" or "protoplasmic process" were mutually identical. At any rate, on the basis of these three observations, he thought that the "protoplasmic process" might be a skeletal mold, derived from one of the primary mesenchyme cells which contained the initial calcareous rudiments. However, he did not dare to push the idea, since he was wondering, on the other hand, whether a geometrical configuration of the primary mesenchyme cells might also play a role in the determination of the skeletal form. It is quite inexplicable why he felt compelled to make a choice between the skeletal mold and the arrangement of the cell as a factor determining skeletal form, instead of trying to consider the interrelation between the two.

PRENANT (1926 a, b) studied skeleton formation in both living and fixed specimens of *Paracentrotus lividus*, *Psammechinus miliaris* and *Echinocardium cordatum*. He found "un système d'anastomoses" among the primary mesenchyme cells in living larvae. The system was a circular tract around the base of the archenteron, with a pair of longitudinal tracts extending toward the apical end (animal pole). From the facts that the formation of the tracts preceded skeletal development and that the skeletal growth invariably followed the course of the tracts, PRENANT placed emphasis on the organization of the tracts as an important factor in the determination of skeletal

form. In cytological studies of fixed materials, he reported that scleroblasts formed an "armature squelettique" by the fusion of their exoplasm, and that crystallization occurred only in the exoplasm, but he failed to explain the relation between these points and the tracts.

BOXIN (1926) reported that a "gaine protoplasmique" was left when the skeleton of the *Paracentrotus lividus* pluteus was dissolved in acidified sea water. He identified the "gaine" with the "tractus" observed by PRENANT.

VON UBISCH (1937) published a comprehensive paper concerning skeleton formation in the larvae of *Echinocyamus pussilus* and *Psammechinus miliaris*. From his observations, the following three facts may be noted. (1) A skeletal rudiment (calcareous granule) is formed within a "Plasmaschicht" which is made up by the fusion of the pseudopodial parts of several primary mesenchyme cells. The "Plasmaschicht" is also termed "ectoplasmatisches Syncytium" or "Plasmasyncytium" because of its cytoplasmic origin. (2) In the course of growth, the tips of triradiate or more advanced spicules are found within a "Plasmastrang", one end of which is continuous with the "Plasmaschicht" and the other end often reaches primary mesenchyme cells situated far from the spicule. (3) When the spicules increase in size, the course of their elongation follows the "Plasmastrang". He emphasized the fact that the growing spicule is, as a rule, enveloped by a "Plasmamantel" which extends beyond the tips of the spicule along a row of primary mesenchyme cells. However, he did not define the relations among the "Plasmaschicht", "Plasmastrang" and "Plasmamantel". Because of this, in spite of detailed observations of them, his description gives a more or less confused impression. It is clear, however, that he was insisting on the presence of organic ground substance as a matrix for the spicule.

Recently, GUSTAFSON and KINNANDER (1956) and RUNNSTRÖM (1957) have mentioned the presence of a structure enclosing the spicule, although without naming it.

This summary may explain why the presence of a skeletal matrix has heretofore not been generally accepted: the chief obstacles appear to be the lack of precise description of the matrix and the dubious attitudes taken by some of the workers themselves regarding the existence of such a structure.

Status of the present investigation

In the previous paper of this series (OKAZAKI, 1956a), it was reported that changes in the calcium concentration of the culture medium affected the form and size of the spicules in sea urchin larvae. Since the results were too complicated to be attributed simply to a deviation in the rate of calcium deposition on the spicule, it was suggested that, given the presence of an organic matrix for

the spicule, effects exerted by the environmental calcium concentration on the form and size of such a matrix might cause corresponding changes to occur in the spicule.

In the present study, therefore, taking the observations of the previous workers into consideration, a concentrated effort was made to find a structure which could be considered as a matrix. From the observations of living larvae of various species, the following four facts have been proved without exception: (1) at the mesenchyme blastula stage (late blastula stage, in which primary mesenchyme cells have already begun to migrate, but gastrulation has not yet set in), a pair of small, sheath-like structures begin to be formed between the ectoderm and a pair of masses of primary mesenchyme cells at two opposing points on the ventral side of the larva; (2) several hours later, a calcareous granular rudiment appears within this structure, which gradually increases in length along a geometrical configuration of the primary mesenchyme cells; (3) the calcareous granule develops into a larval spicule within this structure, which invariably develops a step in advance of the spicule formation; (4) after the calcareous granule has developed into a triradiate or more complicated spicule, the sheath-like structure is observed as strands extending from the tips of the spicule. Some aspects of the structure, at least as they might be described at one developmental stage or another, could be interpreted as the "protoplasmic clump" of THÉEL or the "faint streak", "protoplasmic cord" or "protoplasmic process" of WOODLAND, or the "tractus" of PRENANT or the "Plasma-schicht" or "Plasmastrang" of VON UBISCH.

In the larvae of all the species studied, it was relatively easy to ascertain the presence of this structure. On the other hand, it proved extremely difficult to investigate every detail of the structure because (1) it is transparent and lies in a narrow space between relatively opaque mesenchyme and ectoderm cells, (2) the contour is very complicated with many fine processes projecting out in all directions and (3) the structure readily changes its form if excessively compressed between cover-glass and slide. The inadequacy of the past descriptions is probably due to such circumstances; this could be obviated to some extent by the application of phase contrast and ordinary microscope to *Clypeaster japonicus* larvae, which are highly transparent and have large blastocoels. Observations thus carried

out, and some experimental results, will be reported in the present paper. For convenience, the structure in question will be called "skeletal envelope" in the following pages.

MATERIALS AND METHODS

Materials. For observation of the normal condition, living larvae of five species of sea urchins, *Clypeaster japonicus*, *Hemicentrotus pulcherrimus*, *Mespilia globulus*, *Pseudocentrotus depressus*, *Toxopneustes pileolus* and two species of sand dollars, *Astriclypeus manni* and *Echinarachnius milabilis*, were used. For the purpose of description, however, examples of *Clypeaster* larvae will be selected, because this form gives the clearest details of the skeletal envelope. Evidence has also been obtained proving that the skeletal envelope in larvae of various forms is practically identical to that of *Clypeaster*. For the purpose of the experimentation, *Clypeaster* and *Hemicentrotus* larvae were principally used.

Objective lenses and methods of preparation. For observation of the skeletal envelope, two sorts of oil immersion lenses were found to be satisfactory: F1 (semi-apochromatic lens of Olympus Optical Co.) and DLL (low absorption phase contrast lens of Japan Optical, Inc.).

In preparing the sample, it is important that the larvae be properly pressed between cover and slide, since a slight compression is necessary to obtain a clear microscopic image, while over-compression impairs the skeletal envelope. Procedures suitable for this purpose are as follows: (1) a round drop of an appropriate quantity of larval suspension is placed on the slide in such a manner that the larvae are concentrated at the center of the drop; (2) the cover glass is gently lowered vertically onto the drop so as to spread the larvae evenly. Lowering the cover glass slantingly or removing excess suspension with filter paper after setting the cover glass should be avoided.

Observations must be made as rapidly as possible, since the skeletal envelope gradually changes its form if kept too long under the cover glass.

Experimental media. Ca-low or Ca-high medium: Ca-low medium was prepared by mixing Ca-free sea water (NaCl 26.5 g., KCl 0.7 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 11.9 g., NaHCO_3 0.5 g. plus distilled water to 1000 ml.) and natural sea water in 9:1 ratio; for the Ca-high medium, M/3 CaCl_2 was mixed with natural sea water in 10:45 ratio. Since the resultant calcium concentrations of these mixtures correspond to about $0.1\times$ and $7\times$ the calcium content of natural sea water, these media will be referred to as "0.1 Ca" and "7 Ca", respectively.

Hypotonic sea water: natural sea water was mixed with M/100 CaCl_2 in 7:3 ratio. The resultant mixture has the same calcium concentration as that of natural sea water, while its osmotic pressure is reduced to about $0.7\times$ that of natural sea water. The medium, therefore, will be referred to as "0.7 P".

Acid sea water: this was prepared by adding acetic acid to natural sea water and leaving the mixture to stand overnight so as to stabilize its hydrogen ion concentration by acetate buffer instead of bicarbonate buffer. Since changes in the pH of the acid sea water during the experiment did not exceed 0.2 pH, only the pH value at the beginning of the experiment will be referred to in later descriptions.

RESULTS

Part 1. Normal Process

1. The envelope observed by low powers of ordinary microscope

Before describing the skeletal envelope in detail, it seems appropriate to present low power observations of the geometrical configuration of the primary mesenchyme cells and an outline of the process by which the skeletal envelope develops in *Clypeaster japonicus*. The following terminology will be used hereafter to refer to the continuous changes in the appearance of the special areas under investigation.

For the spicule-forming center:

loose cell assembly → mesenchymal aggregate → skeletal envelope.

For the periphery:

cell assembly → cellular chain → strand.

1) Behavior of the primary mesenchyme cells prior to envelope formation

As is well known, the primary mesenchyme cells arise by successive inward migration of the cells of the blastular wall at the vegetal side. These freed cells are at first crowded at the center of the vegetal wall, until their number reaches about thirty (Fig. 1 A). After this, they begin to disperse along the blastular wall (Fig. 1 B, C), some of them nearly reaching the animal pole (Fig. 1 D).

At the stage shown in Figure 1 D, the primary mesenchyme cells become somewhat more numerous at right and left points on the ventral side (two portions enclosed with dotted line in Fig. 1 D). These groups gradually become larger and more concentrated by drawing in nearby cells to form mesenchymal aggregates (see Fig. 1 E, F and Fig. 2). Simultaneously with the formation of the aggregates, the primary mesenchyme cells nearest the animal pole withdraw a little way toward the vegetal pole (compare Fig. 1 D with F_v),

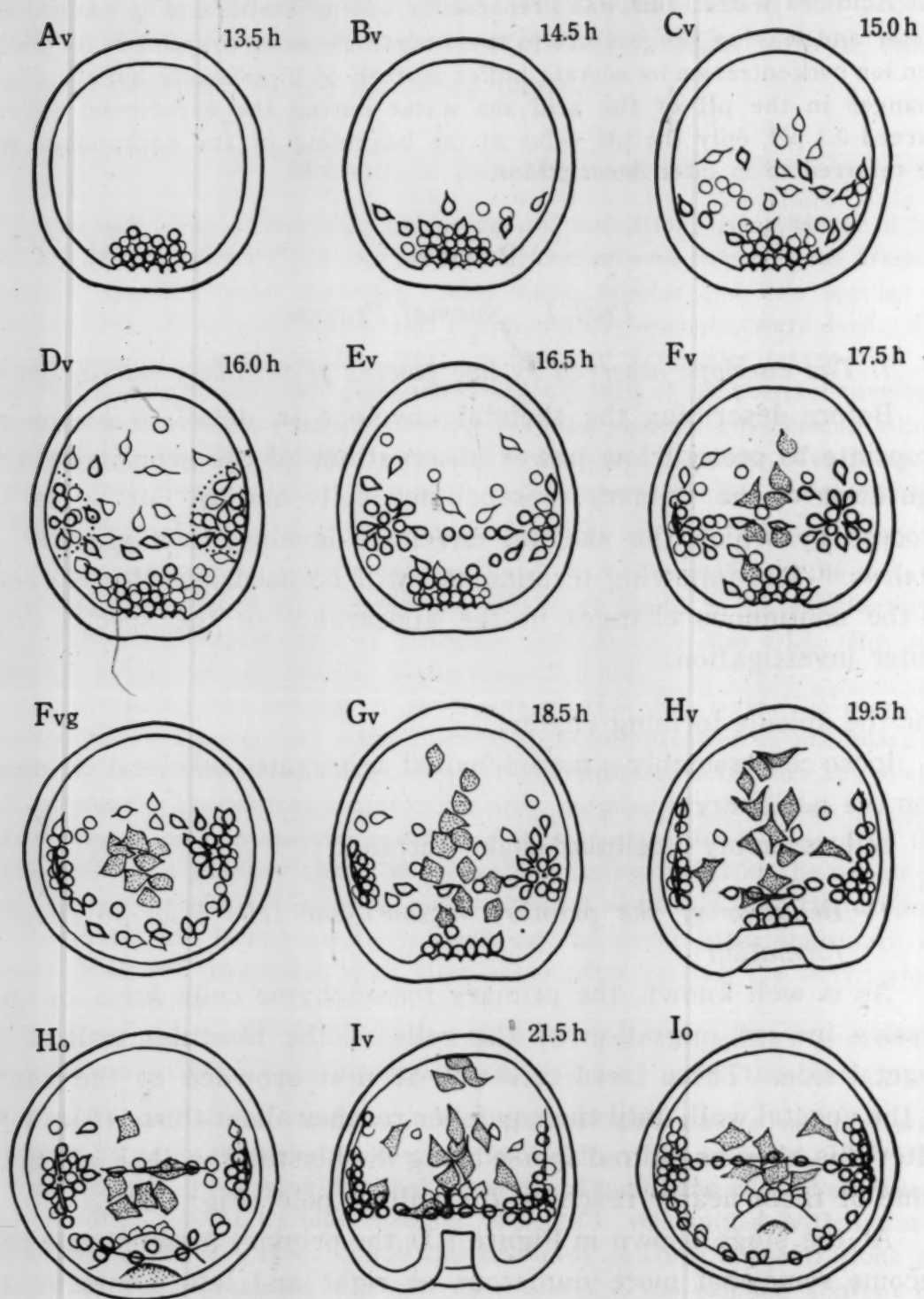


Fig. 1. Camera lucida drawings showing process of development of the spicule and its matrix in *Clypeaster japonicus*. $\times 180$.

Capital letters denote order of developmental stages. Suffixes attached to capital letters: d, dorsal view; o, oblique view; s, side view; v, ventral view; vg, vegetal view. Numeral on each sketch indicates hours after insemination at room temperature (24–25° C.). Primary

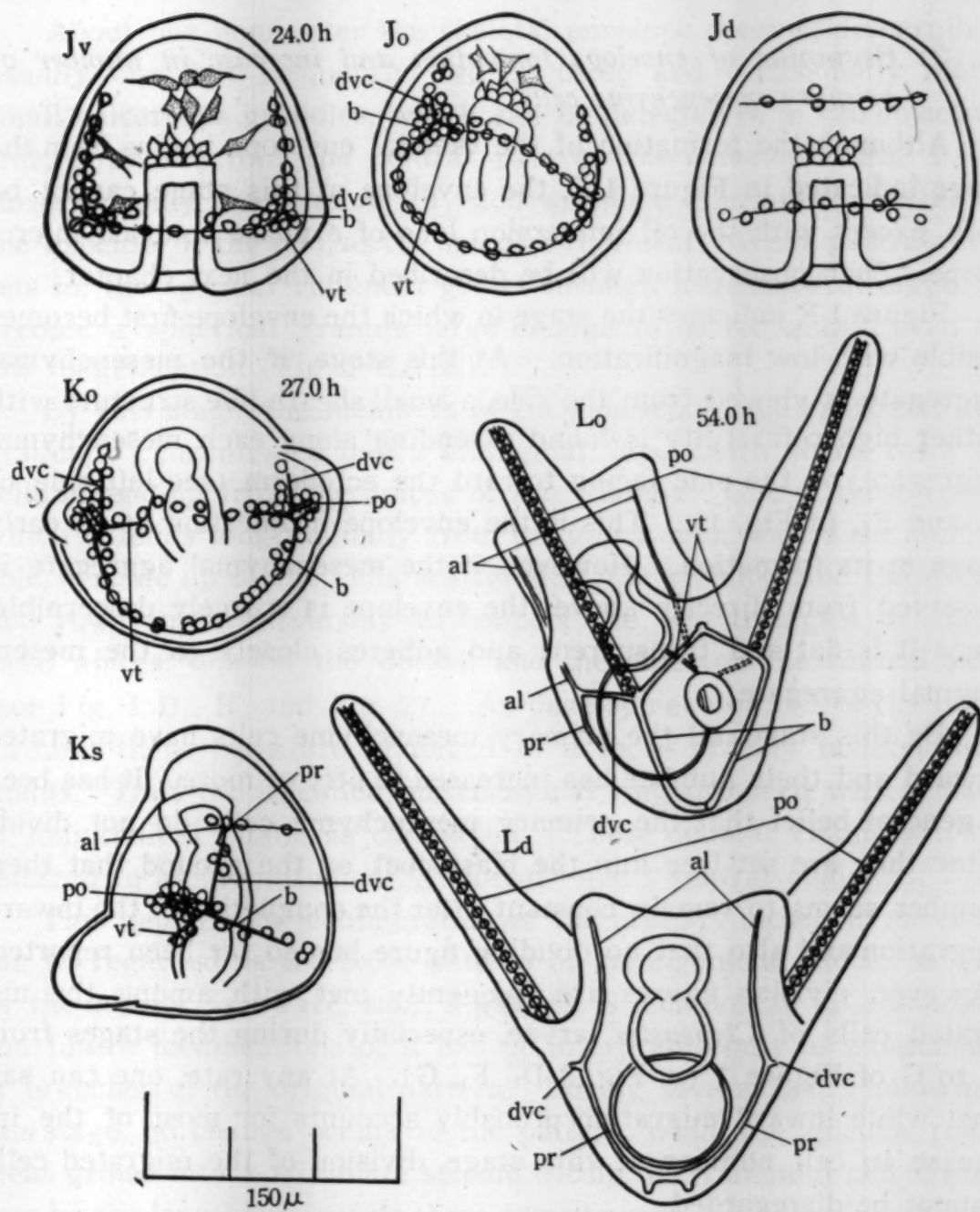


Fig. 1. (Continued)

mesenchyme cells with thick outlines are in focus and cells with thin outlines are out of focus. Stippled amoeboid cells are giant cells (see text).

al, antero-lateral rod; b, body rod; dvc, dorso-ventral connecting rod; po, post-oral rod; pr, primary recurrent rod; vt, ventral transverse rod.

as was pointed out by GUSTAFSON and KINNANDER in larvae of *Psammechinus miliaris* (1956).

2) *Beginning of envelope formation and increase in number of primary mesenchyme cells*

Although the formation of the skeletal envelope begins from the stage indicated in Figure 1 E, the envelope at this stage cannot be seen except with the oil immersion lens of a phase contrast microscope. Such observation will be described in the next chapter.

Figure 1 F indicates the stage in which the envelope first becomes visible with low magnification. At this stage, if the mesenchymal aggregate is viewed from the side, a small sheath-like structure with rather high refractility is found extending along each mesenchymal aggregate, on the side facing toward the ectoderm (see left side of F_v and F_{vg} in Fig. 1). This is the envelope in question at an early stage in its formation. However, if the mesenchymal aggregate is observed from directly above, the envelope is scarcely discernible, since it is flat and transparent and adheres closely to the mesenchymal aggregate.

By this stage, all the primary mesenchyme cells have migrated inward and their number has increased to fifty or more. It has been a general belief that the primary mesenchyme cells do not divide after they are set free into the blastocoel, on the ground that their number seems to remain constant after the completion of the inward migration and also that no dividing figure has so far been reported. However, division figures are frequently met with among the migrated cells of *Clypeaster* larvae, especially during the stages from C to G of Figure 1 (v. Fig. 3 D₂, F₁, G). At any rate, one can say that while inward migration probably accounts for most of the increase in cell number at this stage, division of the migrated cells cannot be disregarded.

Of the 50 primary mesenchyme cells, two groups of from 10 to 15 cells each are forming the mesenchymal aggregates, while about 10 cells on the dorsal side and several cells on the ventral side are distributing themselves along the blastular wall between the two aggregates (see Fig. 1 F_v , F_{vg}). There are still several more cells scattered anteriorly to the above groups.

3) *Appearance of the spicular rudiment and completion of the basic pattern of mesenchymal arrangement*

About one hour after the skeletal envelope becomes discernible, usually one, but frequently two or three and occasionally more, small calcareous granules, which can be detected by a $\times 40$ objective lens, appear within the central part of the envelope (Fig. 1 G). However, only one granule in each envelope continues growth, at the expense of the rest, as the spicular rudiment. When gastrulation sets in, the spicular rudiment grows through a hexahedral stage to become a spherical granule large enough to be recognized even at low magnification (Fig. 1 H_v, H_o).

By this stage, the members of each mesenchymal aggregate are gradually concentrated into a triangular layer with three rows of cells extending from the apices of the triangle. One of these rows, which extends longitudinally from the upper apex toward the animal pole, is made up of the cells scattered in the vicinity, and the other two rows run transversely to connect the two triangles by their basal apices, one on the dorsal, and the other on the ventral side (see Fig. 1 H_v, H_o and Fig. 2). As development proceeds, the cells forming these rows are arranged in a more orderly fashion into chains. Thus the so-called mesenchymal ring provided with a pair of longitudinal chains is completed. These cellular chains will be referred to as "longitudinal", "dorsal" and "ventral chain".

This geometrical configuration of the primary mesenchyme cells can be regarded as the basic pattern of arrangement, since, as far as the author is aware, such a pattern is common to all echinoids and future modifications of it can be interpreted only as extensions or branches of the original pattern. During several hours following this stage, no change occurs in the pattern, while the spicular rudiment grows into a triradiate spicule within the triangular cell aggregate by the lengthening of its three arms toward the apices (compare Fig. 1 H with J). Although the level, as measured from the vegetal wall, where the mesenchymal ring is formed varies among different species, the general statement can be made that the ring is formed close to the vegetal wall in larvae with relatively small blastocoels, while it is removed some distance toward the animal pole in larvae having rather large blastocoels. In *Clypeaster* larvae, the mesenchymal ring is situated about one-third of the blastocoelic

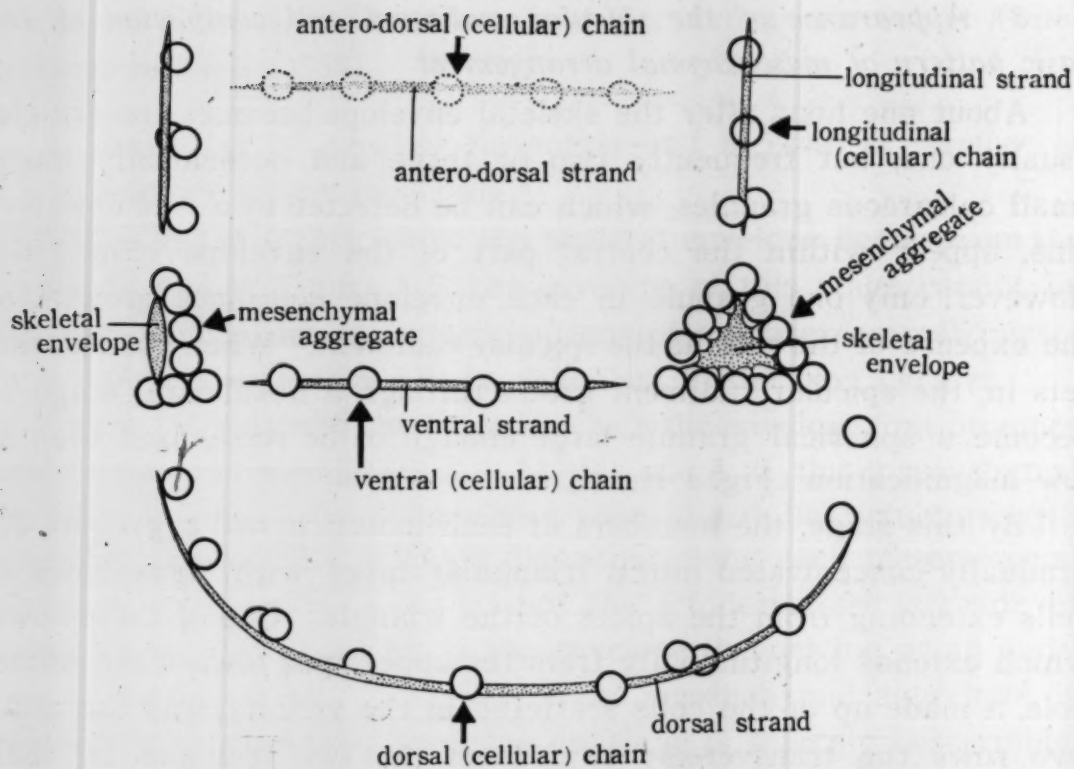


Fig. 2. Diagram representing topographical relationship between envelope system and mesenchymal arrangement. Component cells of antero-dorsal chain and its strand are drawn by dotted lines since this chain is not always observable at the gastrula stage in all species used.

Basic pattern:

mesenchymal ring + pair of longitudinal chains.

Mesenchymal ring:

pair of mesenchymal aggregates + dorsal chain + ventral chain.

diameter toward the animal pole (see Fig. 1 G-J).

Although the few remaining scattered cells have not participated so far in making up the basic pattern, several hours later they form a new chain, "antero-dorsal chain", in parallel with the dorsal chain (Fig. 1 J).

4) Formation of protoplasmic strands along the cellular chains

About the time when the members of the cellular chains have achieved an orderly arrangement a slender protoplasmic strand becomes visible along each chain on the side facing the ectoderm (Fig. 1 H₀). The cells of the chain appear to be attached to their strand, although the connection between them is scarcely discernible with low magnification (the situation will be described in detail in

the next chapter). Although small calcareous granules are frequently found within the strands (v. c.g. in Fig. 3 H₁ and Fig. 4 H), none of these granules increases in size, and sooner or later they disappear. The strands will be named after the cellular chains to which they belong (see Fig. 2). These "strands" seem to correspond to the "protoplasmic cord" of WOODLAND.

5) *Organization of the skeletal envelope*

Early in the formation of the skeletal envelope, its shape can only be guessed to be flat by the fact that the envelope appears as a slender sheath in profile, while its contour is not discernible in a front view (Fig. 1 F). The condition persists for some time, except for the appearance of a spicular rudiment at the center of the envelope.

As the rudiment develops into a triradiate spicule with arms of over ten micra, however, the sheath becomes unrecognizable along the side of the spicule, and is visible only beyond the tips of the arms (cf. Fig. 1 H_v, H_o with I_v, I_o). This may be interpreted as due to the close apposition of the envelope against the side of the spicule, which, in turn, must mean that the contour of the envelope at this stage is triradiate. The portions of the envelope found beyond the spicular tips may be identifiable with the "faint streak" of WOODLAND and the "Plasmastrang" of VON UBISCH.

As was said in the preceding section, the protoplasmic strands have been formed along the cellular chains independently of the spicular envelope (see Fig. 1 H). However, strands and envelope fuse into a single system as they gradually increase in length. In other words, the ends of the longitudinal, dorsal and ventral strands join with the three tips of the envelope, about the time when each arm of the triradiate spicule reaches a length of twenty or more micra (Fig. 1 J). Both ends of the antero-dorsal strand connect with the upper portions of the paired longitudinal strands, as is indicated in the upper portion of Figure 1 K_s. By the prism stage of the larva, therefore, the paired envelopes have been organized into a structure roughly like the skeleton of the pluteus. The envelope so organized may probably correspond to "un système d'anastomoses" of PRENANT, and the strands at this stage are identifiable with the "Protoplasmic process" of WOODLAND.

6) Growth of the spicule

As was mentioned before, the spicular rudiment develops into a triradiate spicule within the envelope closely attached to the mesenchymal aggregate (Fig. 1 G-I), and as the arms of the spicule lengthen beyond the size of the original envelope, they extend into the strands which have joined with the envelope (Fig. 1 J-K). Further elongation of the spicules follows that of the strands, so that the skeletal system of the pluteus stage coincides with the basic pattern of the mesenchymal arrangement of earlier stage. The relation between the names of the strands and those of the resulting skeletal rods is tabulated in Table 1 (v. also Fig. 1 J-L). In the pluteus stage, the right and left skeletal systems are joined into one by the fusion of the body rods, the ventral transverse rods and the primary recurrent rods (Fig. 1 L_o, L_d). It should be noted that while the envelope system is organized by the fusion of several independently formed units, the skeletal system is completed by the uninterrupted growth of the single pair of rudiments.

Table 1. Correspondence between strands of gastrula stage and skeletal rods of pluteus stage

Names of strands		Names of skeletal rods
Longitudinal strand	Proximal to antero-dorsal strand	Dorso-ventral connecting rod (Fig. 1, dvc)
	Distal to antero-dorsal strand	Antero-lateral rod (Fig. 1, al)
Dorsal strand		Body rod (Fig. 1, b)
Ventral strand		Ventral transverse rod (Fig. 1, vt)
Antero-dorsal strand		Primary recurrent rod (Fig. 1, pr)

Here a few words will be devoted to the branching and fenestration of the skeleton, the formation of the post-oral rod being used as an example. Near the junction of the three arms of the triradiate spicules at the stage of Figure 1 J, one process is formed on each arm; these three processes elongate side by side, at the same time forming small lateral spines. These spines fuse, uniting the three processes into a fenestrated rod, the post-oral rod (see Fig. 1 K, L). As the rods lengthen, some of the mesenchymal cells move out of

the aggregate to their tips, suggesting that they are laying down a new section of the envelope (Fig. 1 K_s).

All these facts indicate that the spicules of sea urchin larvae invariably develop within a skeletal envelope, the formation of which follows an orderly arrangement of the primary mesenchyme cells.

7) *Giant mesenchyme cells*

At the stage indicated in Figure 1 F, following the primary mesenchyme cells, about ten large mesenchyme cells migrate into the blastocoel. These cells will tentatively be called giant cells because of their size (stippled cells in Fig. 1 F-J). In the sense that the primary mesenchyme cells are skeletogeneous and the secondary mesenchyme cells are coelom-forming cells, the giant cells do not belong to either of these groups. They are extremely amoeboid, with large lobopodia, and are frequently polynucleate.

Within the blastocoel, they form a cellular column between the animal and vegetal poles (Fig. 1 F, G). As gastrulation sets in and the spicular rudiments begin to be formed, some of these giant cells detach themselves from the column and move to the mesenchymal aggregates or the cellular chains as the case may be (Fig. 1 H, I). There they stretch out and adhere to the cellular groups on the blastocoel side.

The giant cells remaining in the column appear to play a role in gastrulation such as leading the secondary mesenchyme cells toward the animal pole side (v. DAN and OKAZAKI, 1956; OKAZAKI, 1956 b). Only after the gastrulation has proceeded to some extent do they disperse to new positions around the archenteron, where they extend large pseudopodia to span the space between the archenteron and the ectoderm or the primary mesenchyme cells (Fig. 1 J_v).

II. *Detailed observations of the skeletal envelope*

Details of the skeletal envelope observed at higher magnifications will be described in three sections: 1) envelope formation in the mesenchymal aggregate, 2) strand formation in the cell chain and 3) the connection between the envelope and the strands. The alphabetical designation of the sketches in Figure 1 will be used to indicate the developmental stages. The figures in this section (Figs. 3-5) were drawn by superimposing ordinary microscopic and phase contrast images.

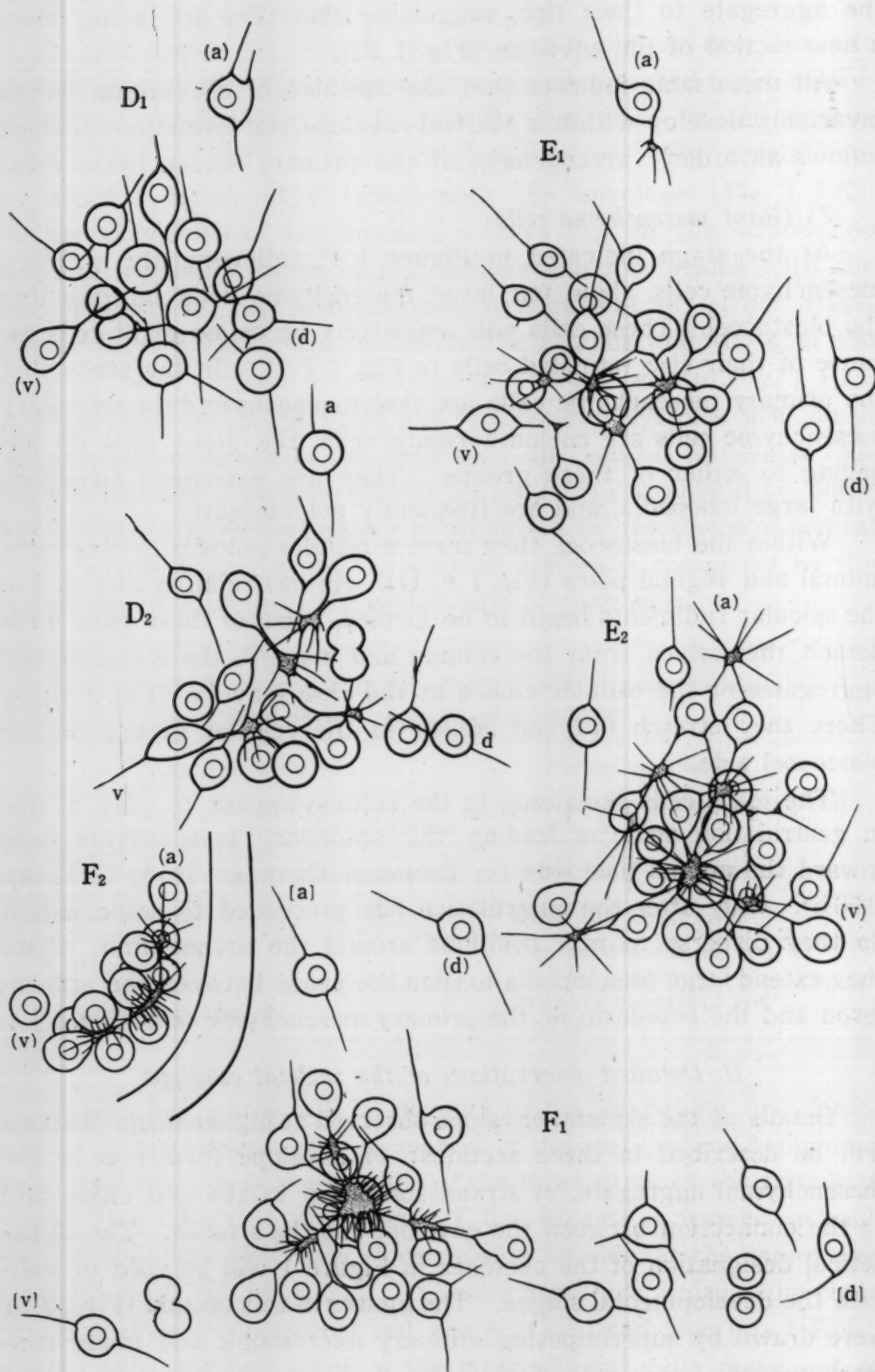


Fig. 3

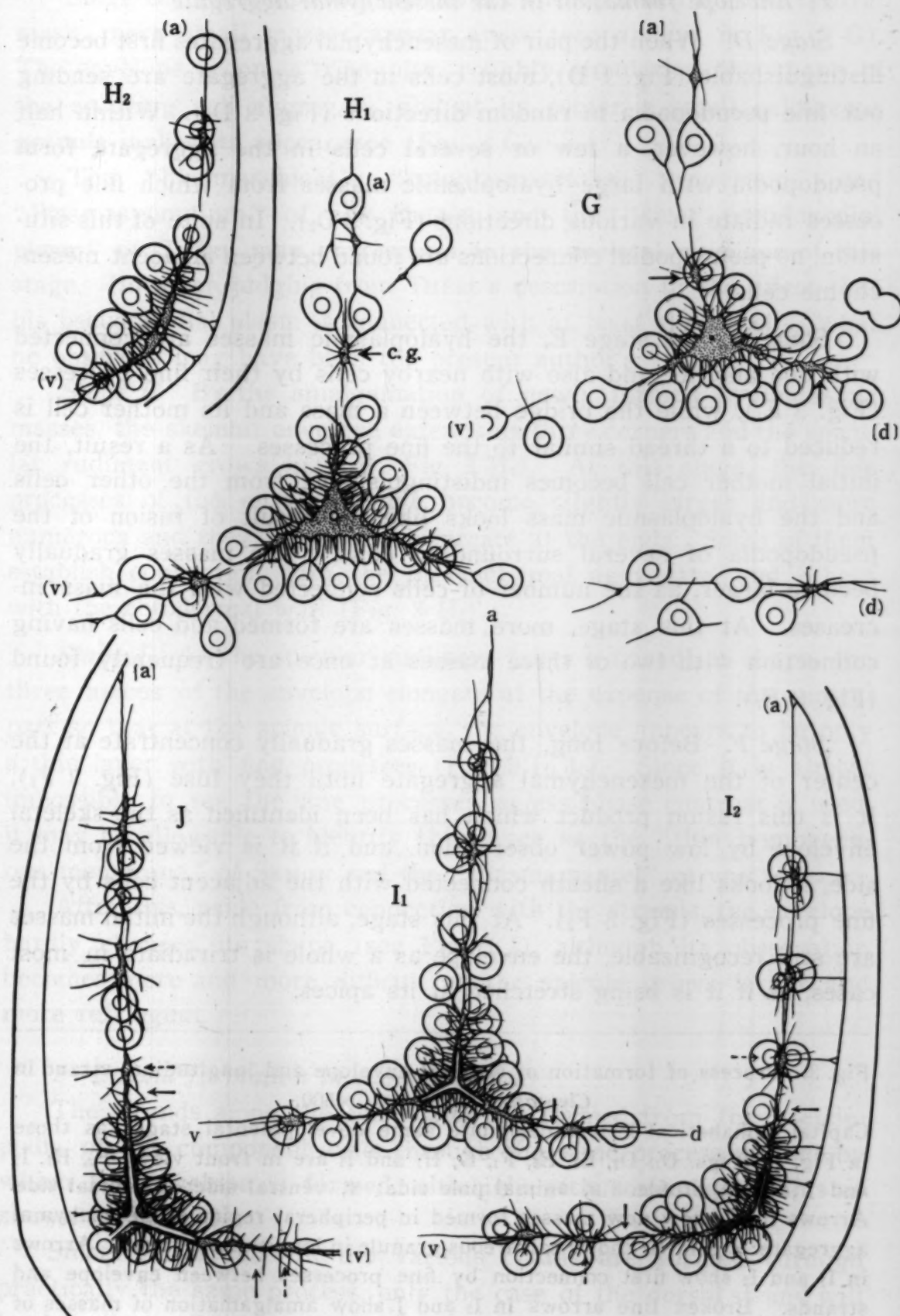


Fig. 3. (Continued)

1) *Envelope formation in the mesenchymal aggregate*

Stage D. When the pair of mesenchymal aggregates first become distinguishable (Fig. 1 D), most cells in the aggregate are sending out fine pseudopodia in random directions (Fig. 3 D₁). Within half an hour, however, a few or several cells in the aggregate form pseudopodia with large hyaloplasmic masses from which fine processes radiate in various directions (Fig. 3 D₂). In spite of this situation, no pseudopodial connections are found between adjacent mesenchyme cells.

Stage E. At stage E, the hyaloplasmic masses are connected with one another and also with nearby cells by their fine processes (Fig. 3 E₁), while the bridge between a mass and its mother cell is reduced to a thread similar to the fine processes. As a result, the initial mother cell becomes indistinguishable from the other cells and the hyaloplasmic mass looks like the result of fusion of the pseudopodia of several surrounding cells. The masses gradually become larger, as the number of cells connected with the mass increases. At this stage, more masses are formed and cells having connection with two or three masses at once are frequently found (Fig. 3 E₂).

Stage F. Before long, the masses gradually concentrate at the center of the mesenchymal aggregate until they fuse (Fig. 3 F₁). It is this fusion product which has been identified as the skeletal envelope by low power observation, and if it is viewed from the side, it looks like a sheath connected with the adjacent cells by the fine processes (Fig. 3 F₂). At this stage, although the initial masses are still recognizable, the envelope as a whole is triradiate in most cases, as if it is being stretched at its apices.

Fig. 3. Process of formation of skeletal envelope and longitudinal strand in *Clypeaster japonicus*. $\times 500$.

Capital alphabetical letters designate same developmental stages as those in Fig. 1. Figs. D₁, D₂, E₁, E₂, F₁, G, H₁ and I₁ are in front view; F₂, H₂, I₂ and J are in profile. a, animal pole side; v, ventral side; d, dorsal side. Arrows in G show new masses formed in peripheral region of mesenchymal aggregate. c.g. in H₁ shows calcareous granule in longitudinal strand. Arrows in I₁ and I₂ show first connection by fine processes between envelope and strands. Broken line arrows in I₂ and J show amalgamation of masses of strands to envelope.

Stage G. As peripheral masses are incorporated into the central mass, more small masses appear anew (see arrows in Fig. 3 G). The envelope becomes triangular, roughly simulating the shape of the encasing cell aggregate, and at its center a small calcareous granule makes its appearance (Fig. 3 G).

The "Plasmaschicht", "ectoplasmatisches Syncytium" and "Plasmasyncytium" of VON UBISCH and the "clear pseudopodial plasm" of THÉEL may correspond to the skeletal envelope of this stage. However, judging from THÉEL's description to the effect that his pseudopodial plasm is connected with at least three cells, what he observed may have been the present author's "strand".

Stage H. By the amalgamation of newly formed hyaloplasmic masses, the skeletal envelope extends its three corners and the spicular rudiment grows in size (Fig. 3 H₁). At this stage, the fine processes of the envelope have become slightly larger and more numerous and they frequently bifurcate at the ends. Some of them establish connection with the mesenchymal aggregate, and others with the ectodermal wall (Fig. 3 H₂).

Stage I. As the spicular rudiment takes a triradiate shape, the three apices of the envelope elongate at the expense of the central part so that at the spicule surface, the envelope appears to be only a thin layer with fine processes (Fig. 3 I₁, I₂). Since it is almost impossible to see the fine processes unless phase contrast is used, it may be allowable to identify this layer as the "thin homogeneous membrane" of SEMON and the "Plasmamantel" of VON UBISCH.

After this, aside from connection with the strands, the envelope hardly changes its shape (see Fig. 3 J), although its observation becomes more and more difficult as the spicule grows larger and more refringent.

2) Strand formation in the cell chain

The strands along the cell chains are formed from the pseudopodia of the component cells through the same process as that by which the envelope is formed, although with some lag. This is shown in Table 2.

Since the strands in the various chains are formed through practically the same process, only the case of the dorsal strand will be described. In the cell chain, several masses which are derived

Table 2. Sequence of formation of envelope and strands in

Successive changes in pseudopodia ↓	Names of cell groups → Stage and hours →	Mesenchymal aggregate	
		Stage	Hrs. after insem.
Appearance of fine pseudopodia		D ₁	15.7
Formation of large pseudopodia with hyaloplasmic masses		D ₂	16.0
Attachment of hyaloplasmic masses to each other and to surrounding cells		E ₁	16.3
Increase in size of masses		E ₂	16.5
Fusion of masses		F	17.5

from the pseudopodia of some of the component cells are linearly distributed, in contrast to the two-dimensional distribution of the masses in the mesenchymal aggregates (Fig. 4 E, F, G). Neighboring masses are subsequently connected with one another by their fine processes, either directly or by the mediation of a cell (Fig. 4 H. v. also Fig. 3 H₁). At this stage, small calcareous granules are frequently found within some masses of the strands (c.g. in Fig. 4 H and Fig. 3 H₁), but these usually disappear later. By stage I, all the masses become directly connected to form the strand and any cells which were mediating between the masses have moved out to the side without losing connection with the masses (Fig. 4 I. see also Fig. 3 I₁). About this time, some of the fine processes are attached to the inner wall of the ectoderm (e.g. Fig. 3 I₂).

Since the fine processes of the masses branch randomly and their branches connect with each other, the resulting strand as a whole forms a network (Fig. 4 I, J). Although the mesh becomes finer and the masses increase in size as development progresses, the masses do not fuse into a single unit, unlike those in the mesenchymal aggregates (cf. Fig. 4 J with Fig. 3 F).

3) The connection between the envelope and the strands

The three strands begin to unite with the three arms of the envelope in Stage I by the fusion of the fine processes (v. arrows in Fig. 3 I₁, I₂ and Fig. 5). After the establishment of these connections, the envelope lengthens its arms by amalgamating the masses of the strands one by one (v. broken-line arrows in Fig. 3

Clypeaster larvae at 24-25° C. See Figs. 3 and 4 for stages.

Longitudinal chain		Dorsal chain		Ventral chain		Antero-dorsal chain	
Stage	Hrs. after insemin.	Stage	Hrs. after insemin.	Stage	Hrs. after insemin.	Stage	Hrs. after insemin.
F	17.5	E	16.5	E	16.5	F-G	18.0
G	18.5	F	17.5	F-G	18.0	H	19.5
H	19.5	G-H	19.0	H	19.5	I	21.5
I-J	21.5	H-J	20.5	I-J	22.0	J	24.0

I, J). In addition, the antero-dorsal strand is connected to both longitudinal and dorsal strands immediately following its formation between Stages I and J (Fig. 5).

On the basis of these observations, it may be concluded that the skeletal envelope, as well as the strands, arise from the pseudopodia of the primary mesenchyme cells, although it is not clear whether all these cells contribute to the completion of the system.

Part 2. Effects of Experimental Conditions

III. Origin of the skeletal envelope

Judging from observations on normal living larvae, it appears that the skeletal envelope arises by fusion of the pseudopodia of several primary mesenchyme cells and continues to increase its size by further addition of the pseudopodial material of other mesenchyme cells. Accordingly, sooner or later, all the primary mesenchyme cells seem to be participating equally in the formation of the envelope. There is, however, a possibility that the envelope is formed by the pseudopodia of several special cells only, and the other cells are simply connected to the envelope without positively contributing material to form it. Facts suggesting this possibility are enumerated as follows:

1) Special cases

In normal cases, when the hyaloplasmic masses unite to form the envelope (Stage E or F), the original pseudopodium connecting a mass to its mother cell has been reduced to fine processes so that

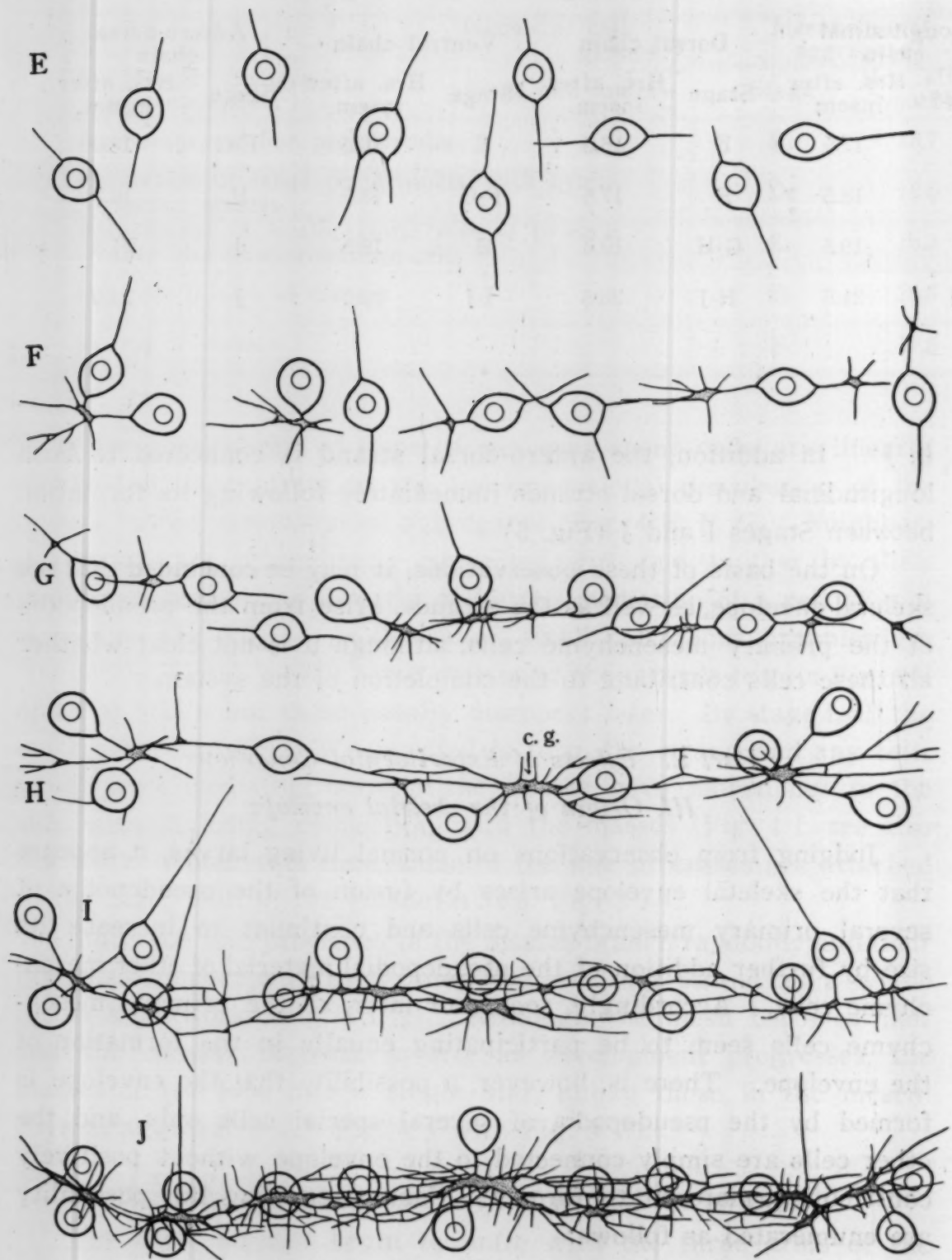


Fig. 4. Details of dorsal strand in *Clypeaster* at various stages. $\times 500$. Capital letters indicate same developmental stages as above.

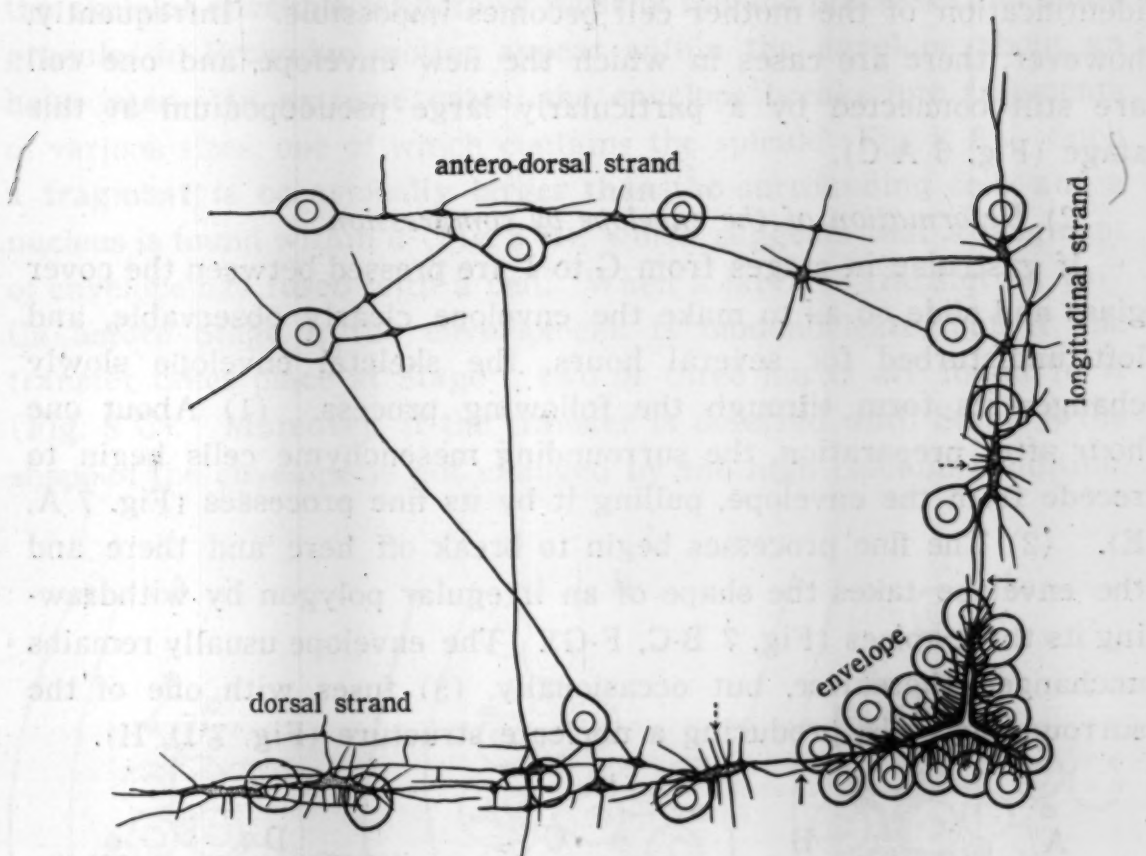


Fig. 5. Connection between longitudinal or dorsal strand and envelope or antero-dorsal strand in *Clypeaster*. $\times 500$. Arrows indicate fine processes connecting envelope to longitudinal and dorsal strand. Broken line arrows show masses which are to combine with envelope at next stage.

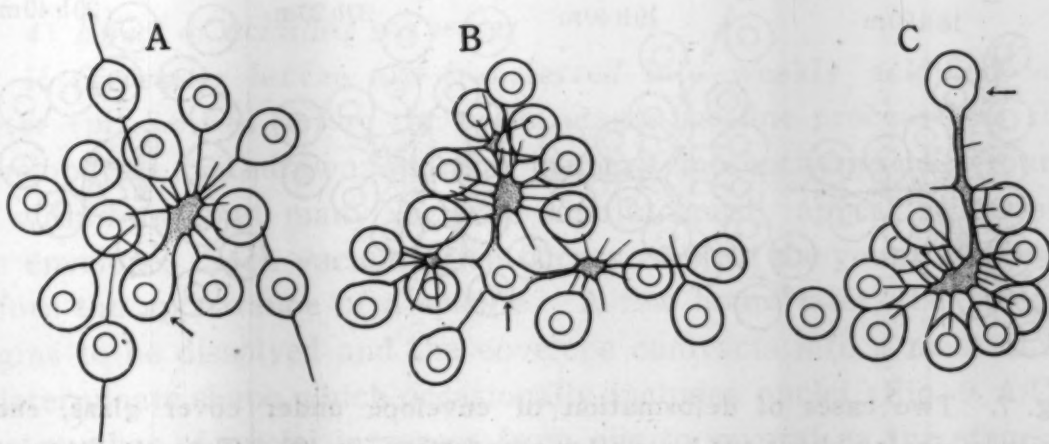


Fig. 6. Tracing of photographs of cases in which envelopes have exceptionally thick connection with one cell (see arrow) in *Clypeaster japonicus*. $\times 500$.

identification of the mother cell becomes impossible. Infrequently, however, there are cases in which the new envelope and one cell are still connected by a particularly large pseudopodium at this stage (Fig. 6 A-C).

2) Deformation of the envelope by compression

If gastrulae in stages from G to I are pressed between the cover glass and slide so as to make the envelope clearly observable, and left undisturbed for several hours, the skeletal envelope slowly changes its form through the following process. (1) About one hour after preparation, the surrounding mesenchyme cells begin to recede from the envelope, pulling it by its fine processes (Fig. 7 A, E). (2) The fine processes begin to break off here and there and the envelope takes the shape of an irregular polygon by withdrawing its three apices (Fig. 7 B-C, F-G). The envelope usually remains unchanged thereafter, but occasionally, (3) fuses with one of the surrounding cells, producing a nucleate structure (Fig. 7 D, H).

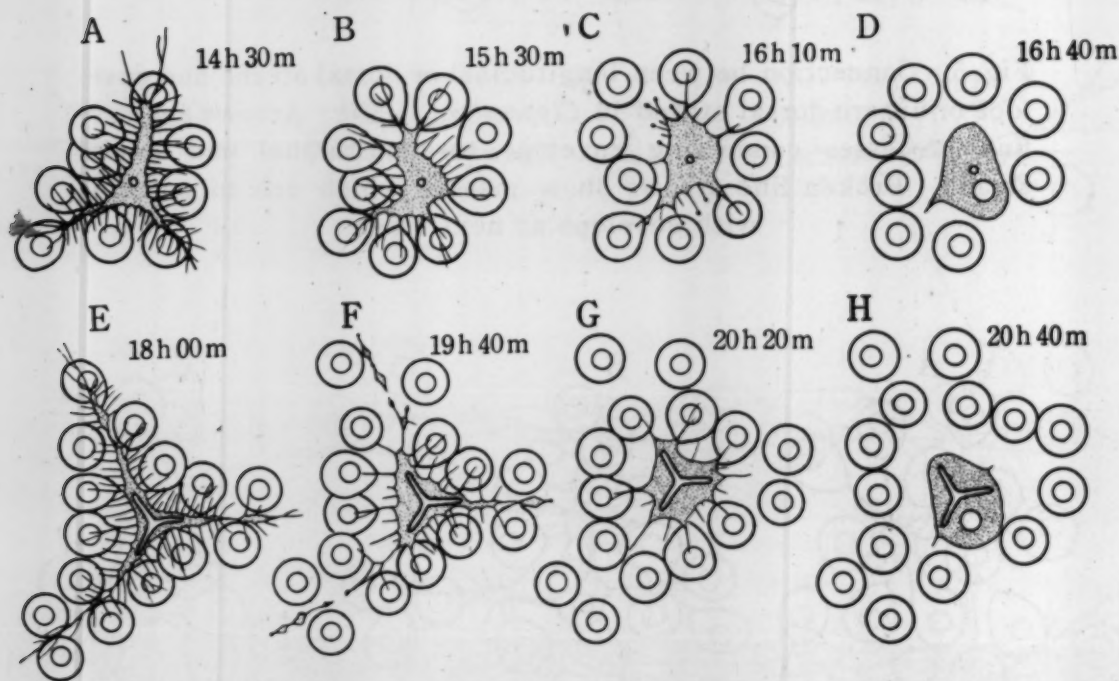


Fig. 7. Two cases of deformation of envelope under cover glass, each series beginning about one hour after preparation. $\times 400$. *Clypeaster japonicus*.

3) Effect of excess calcium

When *Clypeaster* larvae are transferred to 7 Ca before Stage J,

the skeletal envelope withdraws most of the fine processes and many granules in Brownian motion appear within the envelope about an hour later. In extreme cases, the envelope breaks into fragments of various sizes, one of which contains the spicule (Fig. 8 A). Such a fragment is occasionally larger than the surrounding cells and a nucleus is found within it (Fig. 8 B), which suggests that a fragment of envelope has fused with a cell. When a larva is transferred to 7 Ca before Stage I, the envelope-cell is mononucleate, but if the transfer takes place at Stage I, two or three nuclei are found in it (Fig. 8 C). Moreover, if the transfer is deferred until Stage J, the shape of the envelope is not changed by the high calcium condition.

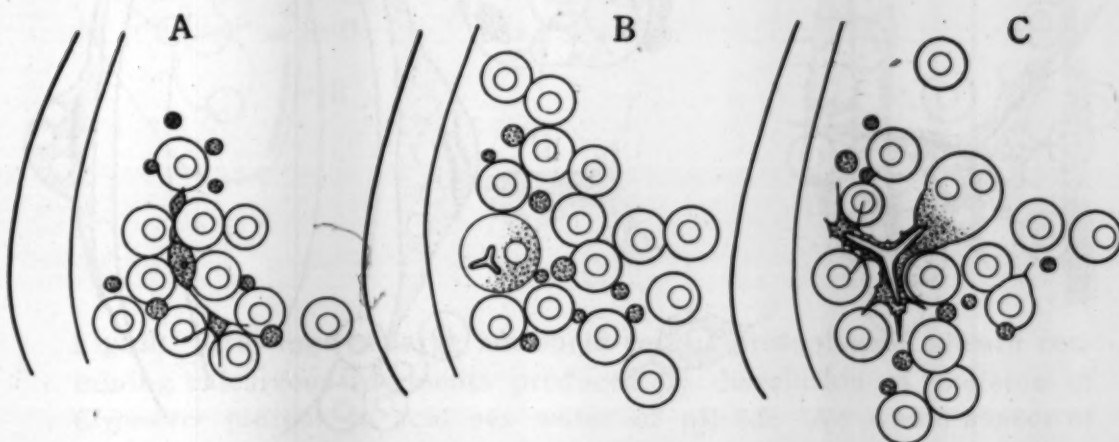


Fig. 8. Deformed envelopes of *Clypeaster* larvae in 7 Ca, traced from photographs. $\times 500$.

4) Effect of acidified sea water

If *Clypeaster* larvae are transferred into weakly acidified sea water (pH 5.6-5.8) before the prism stage, the fine processes of the envelope are withdrawn and the primary mesenchyme cells round up within an hour, many vacuoles simultaneously appearing within the envelope. Such vacuolization occurs even in the young envelope before the appearance of a spicule. A few hours later, the spicule begins to be dissolved and the envelope contracts into a mass of an indeterminate shape which occasionally includes nuclei (Fig. 9 A-C). The number of nuclei increases from one to several as the stage of transfer is deferred.

Although the following was a single observation, it may not be amiss to mention it here. Larvae at the prism stage were exposed

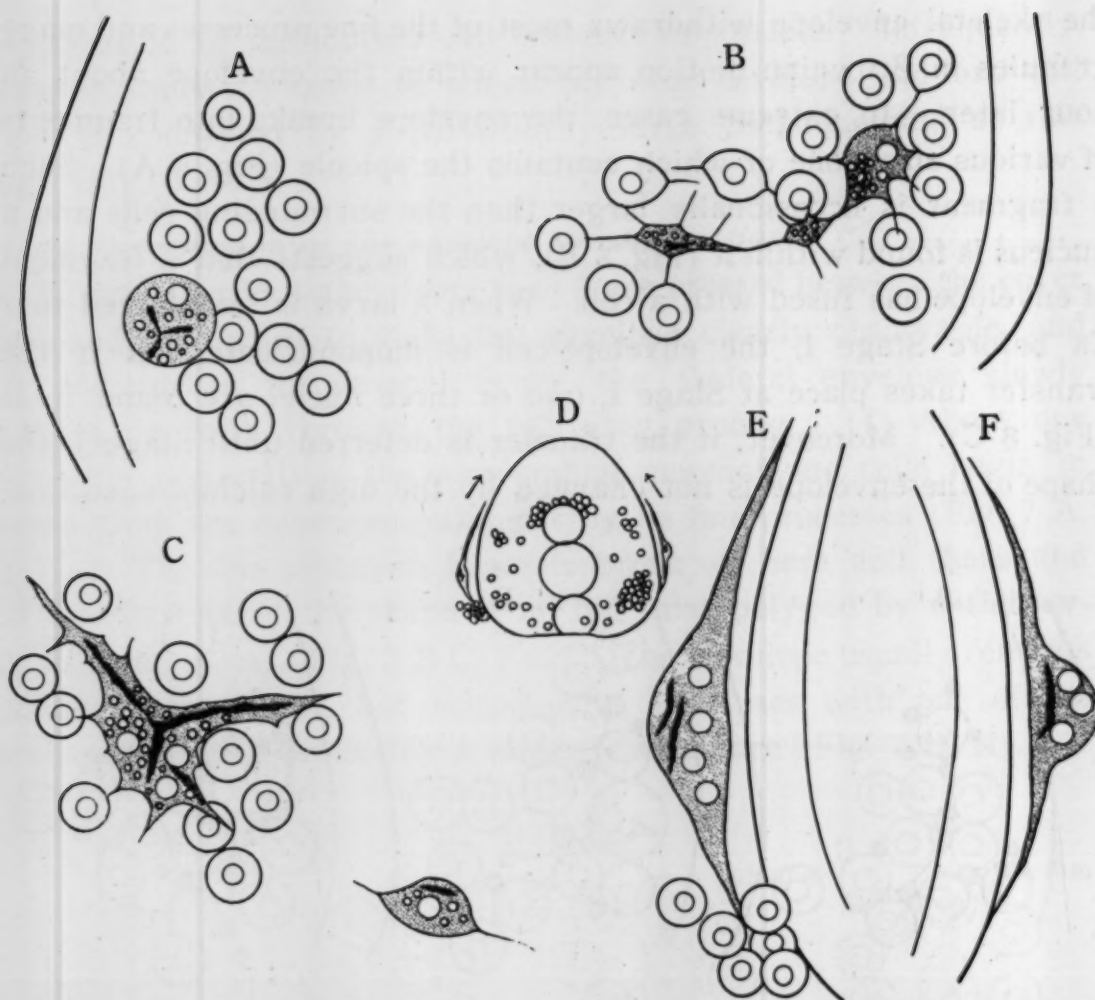


Fig. 9. Tracing of photographs of envelopes of *Clypeaster* gastrulae (A-C) and prism larva (D-F) deformed by transfer to weakly acidified sea water (pH 5.6-5.8). Arrows in D indicate direction of movement of polynucleate structure containing calcareous fragments (see text). E, F: higher magnification of two structures in D. D, $\times 100$; others, $\times 500$.

to the acid sea water (pH 5.6) for six hours, after which they were pressed between the cover glass and slide. The blastular wall of a larva under observation happened to be torn near the post-oral lobe and a mass of mesenchymal cells streamed out of the opening. Before long, two very large polynucleate structures containing calcareous fragments crept out from the cell mass by amoeboid movement and moved around the larva ten or more times in opposite directions for more than two hours, while the other cells in the mass remained motionless (Fig. 9 D-F). These probably result from the fusion of several primary mesenchyme cells with a part of an envelope.

If plutei are transferred to acid sea water (pH 5.5), corrosion of the calcareous skeleton slowly progresses at several places, so that it is divided into pieces of various sizes at about ten hours after transfer (Fig. 10 A). Although such calcareous fragments are usually found within a protoplasmic strand or sheath (Fig. 10 C, E), they frequently lie within amoeboid cells (Fig. 10 B, C, D).

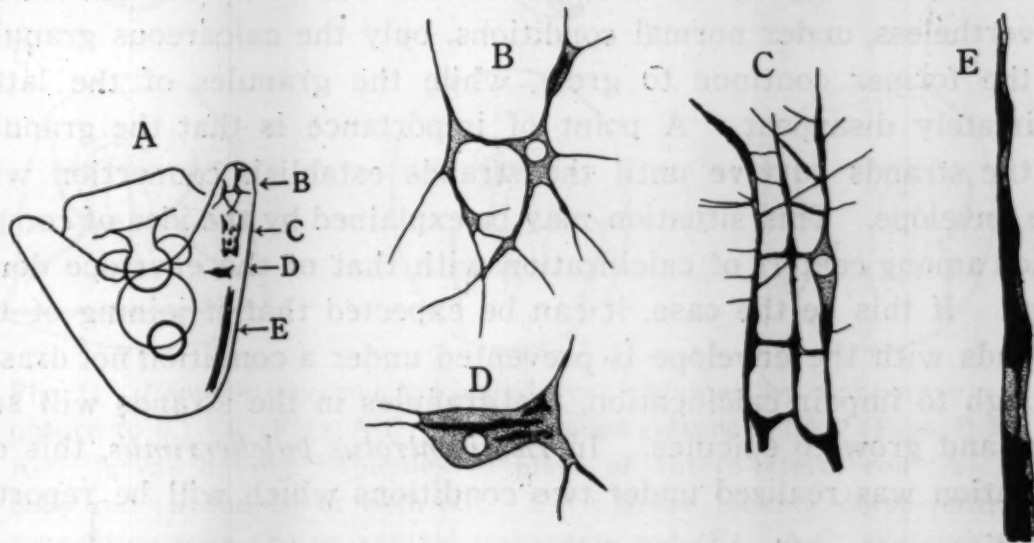


Fig. 10. Drawings of large amoeboid cell or protoplasmic sheath containing calcareous fragments produced by dissolution of skeleton of *Clypeaster* pluteus in acid sea water of pH 5.5. A: whole aspect of pluteus; $\times 100$. B-E: magnified figures of portions of A; $\times 500$.

The phenomena presented in this chapter suggest the possible existence of specialized envelope-forming cells, their reappearance under the unfavorable conditions of the experiments representing a kind of dedifferentiation. Another possibility is not excluded, a few cells might have been fused to the envelope by chance. The giant cells described in Chapter I seem to have a particularly strong tendency for fusion.

As was stated in the historical survey, some previous workers (SELENKA, SEMON, WOODLAND and THÉEL) observed calcareous granules inside the primary mesenchyme cells of normal larvae. In the author's experience, however, the calcareous granule is never found in the cell proper, always lying within the envelope. It seems within the realm of probability that the cells containing calcareous granules observed by the previous workers may have been skeletal

envelopes which had been deformed by the effect of fixatives or some other means.

IV. Relation between calcification centers of strand and envelope

From the standpoint of the capacity for forming calcareous granules and the process by which they are formed, there is no essential difference between the skeletal envelopes belonging to the mesenchymal aggregates and the strands formed by the cell chains. Nevertheless, under normal conditions, only the calcareous granules of the former continue to grow, while the granules of the latter ultimately disappear. A point of importance is that the granules of the strands survive until the strands establish connection with the envelope. This situation may be explained by the idea of competition among centers of calcification with that of the envelope dominant. If this be the case, it can be expected that if joining of the strands with the envelope is prevented under a condition not drastic enough to impair calcification, the granules in the strands will survive and grow to spicules. In *Hemicentrotus pulcherrimus*, this expectation was realized under two conditions which will be reported below.

1) Temporary exposure of larvae to low calcium (0.1 Ca)

As was reported previously (OKAZAKI, 1956a), spicules thicken without lengthening in 0.1 Ca, but on return to sea water, they can grow into the pluteus skeleton. Since this suggests that the basic cause may lie in the effect of 0.1 Ca on the envelope, a further analysis was attempted. Larvae were exposed to 0.1 Ca from various stages between the early mesenchyme blastula and late gastrula (Stages A-J), and returned to sea water after various intervals. The results were observed at the stage when the control larvae developed into plutei (Table 3).

If the larvae are returned to sea water as early as the post-gastrula stage, the skeletal system is completed in much the same manner as in the controls, irrespective of the stage at which exposure is begun.

However, if they are retained in 0.1 Ca until the prism stage of the controls, several spicules develop in the cell chains independently from the spicules in the mesenchymal aggregate, and do not unite with them until later (Fig. 11 A-C). The spicules formed in the

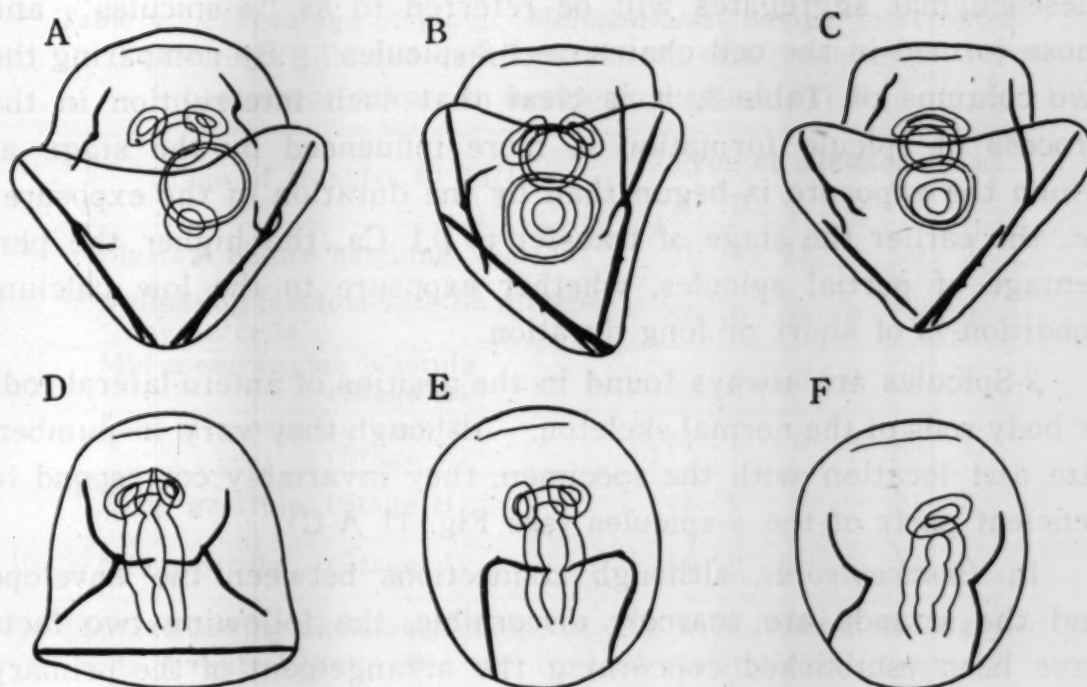


Fig. 11. *Hemicentrotus pulcherrimus* larvae obtained by temporary exposure to 0.1 Ca (Figs. A-C) or continuous culture in 0.7 P (Figs. D-F). A-D: larvae having β -spicules in place of antero-lateral rod (A), of body rod (B and D) or both (C). E-F: larvae lacking dorso-ventral connecting rods (E) or ventral transverse rod (F). A-C: sketches at the pluteus stage of four arms of the control. D-F: sketches at the early pluteus stage of controls. $\times 120$.

Table 3. Interruption of skeleton formation in *Hemicentrotus* larvae exposed to low calcium (0.1 Ca)

Stage of transfer to 0.1 Ca ↓	Stage of return to S. W. →	Prism stage					Early pluteus stage				
	Type of resultant skeleton, hrs. in → 0.1 Ca	Hrs. in 0.1 Ca	Type of skeleton (%)				Hrs. in 0.1 Ca	Type of skeleton (%)			
			A	B	C	D		A	B	C	D
Early mesenchyme blastula (Stage A)		(22)	25	24	25	26	(32)	12	19	26	43
Late mesenchyme blastula (Stage F)		(18)	52	11	31	0	(28)	35	7	54	4
Early gastrula (Stage H)		(16)	75	4	21	0	(26)	53	2	45	0
Mid gastrula (Stage I)		(14)	90	0	10	0	(24)	68	0	32	0
Late gastrula (Stage J)		(10)	98	0	2	0	(20)	97	0	3	0

A, percentage of larvae having complete skeleton. B-D, larvae having interrupted skeleton: skeletal rod in longitudinal chain (B), in dorsal chain (C) and in both (D) are isolated from the central part of the skeleton.

mesenchymal aggregates will be referred to as " α -spicules", and those formed in the cell chains, as " β -spicules". By comparing the two columns of Table 3, it is clear that such interruption in the process of spicule formation is more influenced by the stage at which the exposure is begun than by the duration of the exposure; *i.e.*, the earlier the stage of transfer to 0.1 Ca, the higher the percentage of partial spicules, whether exposure to the low calcium condition is of short or long duration.

β -Spicules are always found in the position of antero-lateral rods or body rods of the normal skeleton. Although they vary in number, size and location with the specimen, they invariably correspond to deficient parts of the α -spicules (see Fig. 11 A-C).

In *Hemicentrotus*, although connections between the envelope and the strands are scarcely discernible, the following two facts have been established concerning the arrangement of the primary mesenchyme cells. (1) In larvae transferred to 0.1 Ca in early stages (Stages A-F), the primary mesenchyme cells which are to form the longitudinal and dorsal chains make several independent cell masses away from the mesenchymal aggregates, and the cellular arrangement corresponding to the ventral chain is not found in later stages. In many of the larvae, this abnormality is not reversed on return to sea water. (2) In larvae transferred later (Stages I-J) to 0.1 Ca, the primary mesenchyme cells maintain their initial arrangement for a considerable period, although they ultimately become disordered, showing greater stability of arrangement as the stage of transfer is deferred. On the basis of these facts, the observed interruption of the process of spicule formation in 0.1 Ca can be explained by thinking that union of the strands with the envelope fails in 0.1 Ca and if the exposure is long enough, such union is permanently blocked even after subsequent return to sea water, which would permit continued independent growth of the calcareous granules in the strands. On the other hand, if the strands have joined with the envelope before the transfer, the envelope system resists fragmentation, and calcification in the strands will be suppressed.

2) Continuous culture of larvae in hypotonic medium (0.7 P)

β -Spicules are formed in place of the ventral transverse or body

Table 4. Percentage of plutei (*Hemicentrotus*) having interrupted skeleton (B-D) and deficient skeleton (E-F) following continuous culture in hypotonic medium (0.7 P)

Stage of transfer to 0.1 Ca	Type of skeleton (%)					
	A	B	C	D	E	F
Blastula before hatching	21	4	71	4	0	0
Swimming blastula with no mesenchyme cells	67	0	33	0	0	0
Mid mesenchyme blastula (Stage D)	85	0	2	0	9	4
Late mesenchyme blastula (Stage F)	64	0	0	0	28	8
Early gastrula (Stage H)	77	0	0	0	21	2
Mid gastrula (Stage I)	84	0	0	0	13	3

Larvae having skeletons: complete (A), with isolated skeletal rods in ventral chain (B), with isolated skeletal rods in dorsal chain (C), in both (D), lacking dorso-ventral connecting rod (E), lacking ventral transverse rod (F).

rods when larvae are continuously cultured in 0.7 P from as early as the blastula stage, before the appearance of the primary mesenchyme cells (Table 4). When the control larvae develop to the prism stage, both ends of the dorsal strands of such experimental larvae are frequently connecting with the ectodermal wall instead of with the mesenchymal aggregates. In such cases, small calcareous rods, one, two, or occasionally three, are found in the dorsal chain, suggesting that the dorsal strand is further subdivided. If the larvae remain in the experimental media longer, the calcareous rods in the dorsal chain develop into skeletal rods so large that the posterior portion of the pluteus frequently becomes squarish (Fig. 11 D) or asymmetrical (much the same as Fig. 11 B). β -Spicules are often found in the ventral chain but these do not develop to so large a size and retain the triradiate form. At all events, when β -spicules are formed, a corresponding deficiency is invariably found in the α -spicule, as in 0.1 Ca.

When larvae are transferred into 0.7 P after the appearance of the primary mesenchyme cells, β -spicules are seldom formed. However, the skeletons of the experimental larvae are frequently found to be underdeveloped in one component rod—either the dorso-ventral connecting rod or the ventral transverse rod, while the body

rod is least affected (Fig. 11 E, F; Table 4).

Comparison of the results of early and late transfer, reveals a seemingly paradoxical situation. The fact that early transfer leads to the formation of β -spicules as well as α -spicules may mean that although quasi-normal cell chains are formed, the connection of the strands with the envelope is disturbed. On the other hand, the formation of α -spicules with missing components and without supplementary formation of β -spicules following late transfer may mean either that the cell chains are not formed at all, or that even though they are formed, they cannot function. A possible way out of this difficulty is to assume thinking that in the early transfer experiments, the primary mesenchyme cells have enough time for accommodation to the new medium before they form the chains, but with late transfer, the shock is received while they are in the midst of this process.

On the basis of these experimental results, the following three statements may be made. (1) Primarily, the potency to form pluteus skeleton is possessed by both the envelope and the strands. (2) Under normal conditions, however, the rod-forming function of the strands is inhibited by the envelope, to which the strands connect themselves. (3) If the strands are prevented from joining the envelope, this inhibition does not operate, and the calcareous granules within the strands survive and continue to grow.

In earlier studies of sea urchin larvae, numerous experimental conditions have been reported as bringing about spicule abnormalities. Among these there are cases in which several spicules develop simultaneously in a larva (HERBST, 1895, 1897; PRENANT, 1926 a, b; HÖRSTADIUS, 1936 a, b, 1957; HÖRSTADIUS and GUSTAFSON, 1954; LINDAHL, 1936; LALLIER, 1955; RULON, 1955; OKAZAKI, 1956 a; BEVELANDER and NAKAHARA, 1960 etc.). Such a result might be brought about by the separation of the envelope and the strands or by subdivision of either or both of them.

V. Skeletal envelope as organic matrix of spicule

From the foregoing considerations, although there is no doubt that the spicules of sea urchin larvae invariably grow within the envelope, it is still a question whether the envelope is a mere outer covering of the spicule or acts as the ground substance on which

mineral components are deposited. In an attempt to answer this question, spicules were dissolved by acid and the following evidence obtained in support of the latter alternative.

1) BOXIN (1926) showed that if *Paracentrotus plutei* were transferred to acid sea water, their skeletons were dissolved without killing the larvae, leaving sheaths of the same shape as the skeletons. The present author confirmed and extended BOXIN's results, using acid sea water of pH 4.5-5.0 in all the species mentioned above. Although the skeleton shows strong birefringence, the sheath itself is isotropic. If *Hemicentrotus* or *Pseudocentrotus* larvae are used, the interior of the sheath is frequently tinged with orange-red, indicating that some organic substances are contained in the sheath.

2) If gastrulae with small triradiate spicules, such as that shown in Figure 3 I, are transferred to acid sea water of pH 5.0-5.2, their spicules are dissolved within half an hour, prior to the deformation of the envelope. During this dissolution process, no trace of any sort is left within the envelope where the spicule had been, suggesting that calcium is deposited on an organic substratum of uniform mesh.

3) If a spicule is composed of such an organic substratum and calcium salt which is deposited on it, the former should remain even when isolated spicules deprived of their envelopes are dissolved by acid. Spicules were isolated by the following procedure: larvae concentrated by a hand-centrifuge were washed a few times with distilled water of pH 8.0 controlled by the addition of sodium hydroxide, fifteen times volume of M/5 NaOH was added and the suspension incubated at 100° C. for 30 minutes to dissolve the cytoplasm completely. Pretreatment with distilled water (pH 8.0) is indispensable for complete removal of the cytoplasm. Finally, the spicules were washed several times with distilled water until the supernatant fluid was no longer colored by phenolphthalein. Spicules thus isolated have no covering whatsoever.

If M/100 acetic acid or hydrochloric acid is added to the isolated spicule from the side of the cover glass, the calcareous component of the spicule soon disappears and a cord of the same shape is left behind. The cord is difficult to see without the aid of staining or phase contrast. It was found that Nile blue sulphate stained the cord blue but not the spicule, so that if the dye is added during

preparation, the stained ground substance becomes apparent as the calcareous component of the spicule is dissolved away.

On the basis of the above observations, it can be said that the skeletal envelope of sea urchin larvae functions as an organic matrix for the spicule; that is, the growth of the spicule is not a purely physical crystallization of calcium carbonate within the envelope but a successive arraying of calcareous elements onto an organic substratum.

DISCUSSION

From the results reported in this paper, it can be concluded that one of the important roles of the primary mesenchyme cells is to form the skeletal matrix by their pseudopodial material. In the mouse, it has been demonstrated from radioautographic analysis that osteoblasts or odontoblasts build the precursor of the matrix in their cytoplasm but have no connection with the transfer of calcium ions (KUMAMOTO and LEBLOND, 1956; CARNEIRO and LEBLOND, 1959). On the other hand, NAKANO (1960) has recently reported a rapid increase in incorporation of Ca^{45} into sea urchin larvae at the mesenchyme blastula stage before spicule formation, suggesting accumulation of calcium salts by the primary mesenchyme cells, and BEVELANDER and NAKAHARA (1960) emphasize that the primary mesenchyme cells act in both accumulation of Ca ions and formation of an organic matrix. The investigation of this point in sea urchin larvae will be the subject of future research.

Incidentally, in the same paper, BEVELANDER and NAKAHARA arrived at a rather queer conclusion which happened to be practically identical with that of SEMON (1887). They report that in *Echinarachnius parma*, the initial step of spicule formation occurs intracellularly but subsequent growth of the spicule proceeds extracellularly "upon or within a delicate fibrillar matrix which was formed by the mesenchymal cells as a pseudopod-like structure" (p. 47). Concerning the initial step, they write that spicular rudiments are formed in a pair of mesenchymal cells which are "somewhat elongated and irregular in shape with profusion of vacuoles". They appear to believe that the vacuoles are indispensable for spicule formation. Although the subject of normally vacuolated envelopes has not been touched upon in the present paper, since

descriptions are restricted to *Clypeaster japonicus*, vacuolization of the envelope is frequently met with in *Mespilia globulus*. From the author's standpoint, the structure which BEVELANDER and NAKAHARA refer to as an elongated and vacuolated cell could easily be the superimposed figures of the author's so-called envelope and an underlying mesenchyme cell. This is particularly likely, since all the cells within a given mesenchymal aggregate are observed to be round at the beginning of spicule formation, and further, a typical contour of an envelope can be traced in the photograph shown by these workers (BEVELANDER and NAKAHARA, 1960 Fig. 6). They further extended their study to radioautographic analysis and to experimental production of multiple spicules. In their radioautograph, the areas darkened by Ca^{45} , one of which is interpreted as a spicule and the other as primary mesenchyme cells, correspond more closely in shape and position to the skeletal envelope of the author (cf. Chapter II). The formation of multiple spicules, which they explain by imagining that many cells are capable of producing spicules, can as well be covered by the idea that the strands fail to unite with the envelopes (cf. Chapter IV).

Since the matrix of the sea urchin larva is derived from the pseudopodia of the primary mesenchyme cells, the skeletal matrix, in a sense, is part of these cells. On the other hand, the matrix is physiologically different from the surrounding cells, as is shown by its characteristic formation of fine granules in high calcium and vacuolization in weakly acidified sea water, neither of which occurs within the cells (Sections 3 and 4 of Chapter III). In short, if the matrix is considered to be a part of the mesenchyme cells, the position of the spicule is intracellular, but if it is thought to be independent of these cells, the position of the spicule will become extracellular all through its development. But a change from one to the other as suggested by SEMON, and BEVELANDER and NAKAHARA, is extremely difficult to imagine.

Since the matrix is closely in contact with the primary mesenchyme cells, it is natural that the shape of the matrix, and consequently the course of elongation of the spicule, must follow the arrangement previously set by the primary mesenchyme cells. What, then, is the factor which governs the arrangement of the primary mesenchyme cells? It has been known for some time that the

mesenchymal ring is moved away from the vegetal body wall in proportion to the degree of vegetalization of the larva, in accordance with the double gradient theory (HÖRSTADIUS, 1939). RUNNSTRÖM (1929) and VON UBISCH (1937) have expressed the opinion that a factor governing the form of the spicule lies in the ectoderm. In the course of the present study, data have been accumulated showing a correlation between a locally limited characteristic morphology of certain ectodermal cells and the position of the mesenchymal ring (OKAZAKI and FUKUSHI, in preparation).

Although it has been shown that the skeletal matrix is derived from the pseudopodia of the primary mesenchyme cells, the question is still open as to whether all the primary mesenchyme cells or only some specialized cells contribute their pseudopodial material to form this matrix. A method is currently being worked out to settle this point.

SUMMARY

1. The first step in the process of spicule formation in sea urchin larvae is arrangement of the primary mesenchyme cells in an orderly pattern composed of a pair of mesenchymal aggregates and dorsal, ventral and paired longitudinal cell chains.

2. As the second step, a skeletal matrix which results from fusion of the pseudopodia of the primary mesenchyme cells is formed within each of these cellular groups. The skeletal matrix system of the larva is thus completed by the fusion of separate matrices.

3. The third step is the appearance of a triradial spicule within each of the paired matrices of the mesenchymal aggregates. The growth of the spicule takes place by the uninterrupted elongation of its three arms through the matrix system, each triradial spicule giving rise to half of the pluteal skeleton.

4. The matrix system of the whole body, on the other hand, is united into one unit. Consequently, the two half skeletons are eventually united on the median plane, completing the skeletal system of the pluteus.

5. If the fusion of the separate matrices is experimentally prevented, each of them forms a part of the pluteus skeleton which remains unconnected with the rest.

6. Judging from the observation in normal larvae, all the primary

mesenchyme cells appear to contribute to the formation of the matrix, but some experimental results suggest the presence of specialized matrix-forming cells.

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THE EFFECT OF AUREOMYCIN ON DEVELOPMENT OF THE FROG¹⁾

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Numerous investigators have shown that when minute quantities of aureomycin (chlortetracycline), terramycin (oxytetracycline), penicillin or streptomycin were fed together with the basal ration to chicks, swine, cows and other domestic animals they gained body weight (JUKES and WILLIAMS, 1953; STOKSTAD, 1954 and JUKES, 1955). The growth-promoting effect of antibiotics is postulated to result from changes of intestinal microflora produced by the antibiotics (JUKES, 1955). The present experiment was planned to determine the effect of an antibiotic on the growth of frog tadpoles during metamorphosis and to consider the possible mechanism of the effect, if any.

MATERIAL AND METHODS

Ninety tadpoles of *Rana catesbeiana*, raised from eggs obtained from a single pituitary-injected female, were divided into three groups at the metamorphic stages IV and V (TAYLOR and KOLLROS, 1946). The first group of tadpoles was fed with control food consisting of 65% of wheat flour, 33% of whole duck's egg and 2% of dry yeast powder. The second and third groups were fed the same food containing 10 g. and 1 g. of aureomycin (Lederle) per 10 kg. of control food respectively. In addition, to each 10 kg. of the two kinds of aureomycin food 1 mg. of vitamin B₁₂ (Lederle) was added in order to obtain the maximal growth effect. This was done because the first preparation of antibiotics used widely in animal feeds contained aureomycin and vitamin B₁₂. It was thought then there was some interrelationship between the antibiotic and vitamin B₁₂.

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on the growth-promoting effect. For CRAVIOTO-MUNOZ *et al.*, 1951 and DAVIS *et al.*, 1951 reported that aureomycin might increase the availability of vitamin B₁₂ while vitamin B₁₂ was shown to influence protein synthesis (HARTMAN *et al.*, 1949 and CHARKEY *et al.*, 1950).

The three groups of tadpoles were reared in separate aquaria each containing 30 liters of tap water and some green aquatic plants. Food was given daily and the excess was removed the next day. The water was changed twice weekly. The tadpoles were kept under identical conditions with regard to space, temperature, light and food (except its constituents). Stages of metamorphosis were observed once a week under a binocular dissecting microscope, and the total length from the snout to tip of the tail and body weight were measured.

Another experiment was repeated feeding with aurofac (Lederle), a commercial product of crude aureomycin, every pound of which contains 1.8 g. of aureomycin and 1.8 mg. of vitamin B₁₂. For this, 70 tadpoles, artificially inseminated from the same pair of bullfrogs used in the previous experiment, were divided into two groups, at stage VII; one was fed control food, the other with aurofac food which was made by mixing 2.5% of aurofac by weight to the control food so that it contained 1/10,000 aureomycin.

RESULTS

I. Aureomycin experiment

1. Progress of metamorphosis—The rate of metamorphosis as measured by the developmental stages of the growth of hind legs (TAYLOR and KOLLROS, 1946) for eleven consecutive weeks is shown in Fig. 1.

It is obvious that the growth rate of the hind legs was greater in the treated tadpoles than the controls, and it seemed that the 1/10,000 aureomycin food produced a greater effect than did the 1/1,000 aureomycin food. The differences of the metamorphic stages among the different groups of tadpoles were small at first and then became marked as metamorphosis approached the end.

By testing the statistical significance of mean differences of the metamorphic stages among the three groups of tadpoles with WELCH's *v*-method (WANG, 1955, this method is more critical than that of

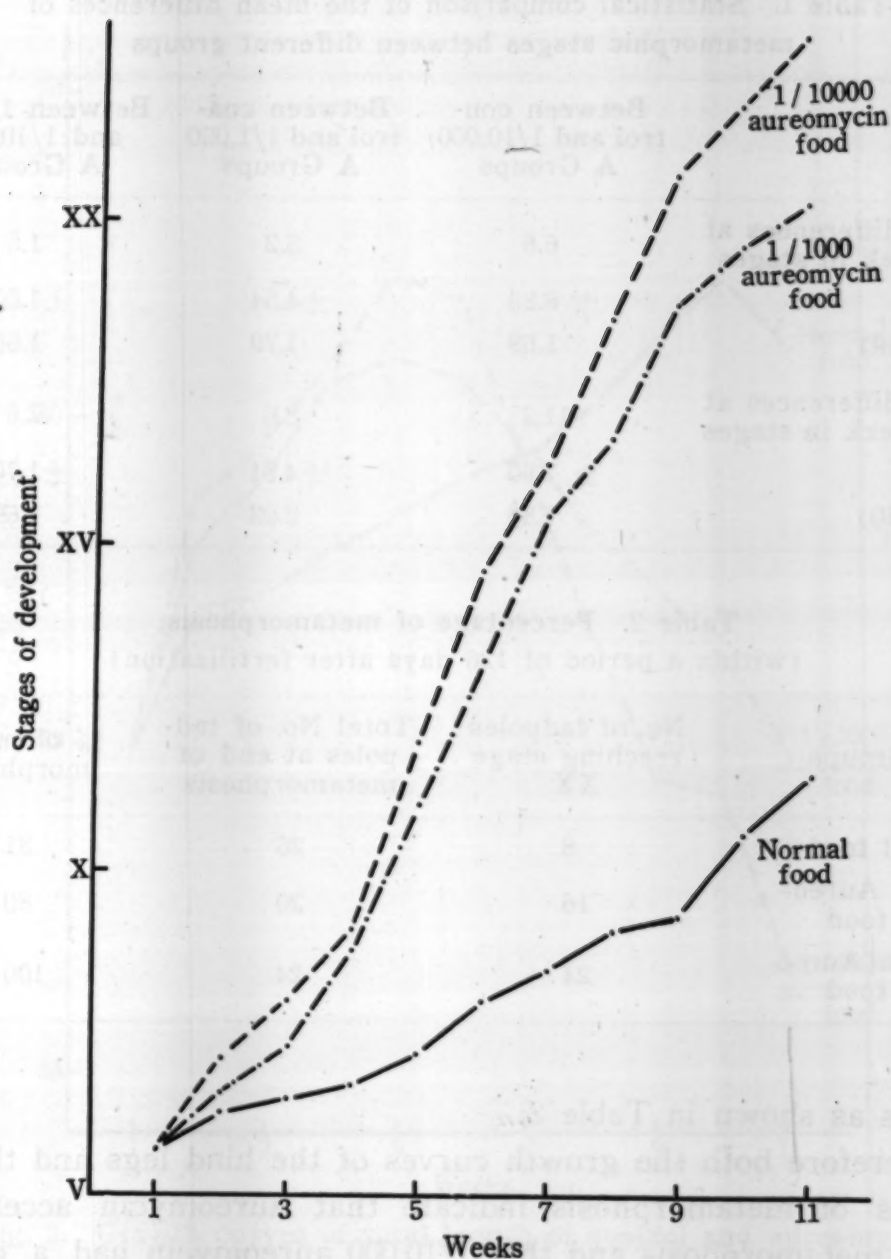


Fig. 1. Growth curves of the metamorphic stages of control and aureomycin fed tadpoles.

STUDENT'S *t*-criterion when variabilities of the two samples are different), it was found that the mean differences are statistically significant, for *v* is greater than *t* in every case except the 6th measurement between 1/1,000 and 1/10,000 groups as shown in Table 1.

The effect of acceleration on metamorphosis was further observed among the percentages of metamorphosis of the three groups of

Table 1. Statistical comparison of the mean differences of metamorphic stages between different groups

	Between control and 1/10,000 A Groups	Between control and 1/1,000 A Groups	Between 1/1,000 and 1/10,000 A Groups
Mean differences at 6th week in stages	6.6	5.2	1.5
v	± 6.25	± 4.54	± 1.00
$t(p=0.10)$	1.69	1.70	1.68
Mean differences at 11th week in stages	11.2	8.6	2.6
v	± 7.60	± 4.81	± 1.70
$t(p=0.10)$	1.68	2.02	1.69

Table 2. Percentage of metamorphosis (within a period of 126 days after fertilization)

Groups	No. of tadpoles reaching stage XX	Total No. of tadpoles at end of metamorphosis	% of metamorphosis
Control food	8	26	31
1/1,000 Aureomycin food	16	20	80
1/10,000 Aureomycin food	24	24	100

tadpoles as shown in Table 2.

Therefore both the growth curves of the hind legs and the percentages of metamorphosis indicate that aureomycin accelerated tadpole metamorphosis and that 1/10,000 aureomycin had a greater effect than 1/1,000 aureomycin.

2. *Development of total length*—Aureomycin in the present experiment affected the rate of metamorphosis, but did not influence the total length of tadpoles. In Figure 2 it is seen that the aureomycin-fed tadpoles reached their maximal total length five weeks earlier than did the controls. Statistically the average maximal total lengths of the control (64.05 mm.) and of the 1/10,000 aureomycin fed tadpoles (64.32 mm.) are not proved to be significantly different from each other (with a mean difference of 0.27 mm. where $v = \pm 0.15$ and $t = 1.67$ at the standard of $p = 0.10$).

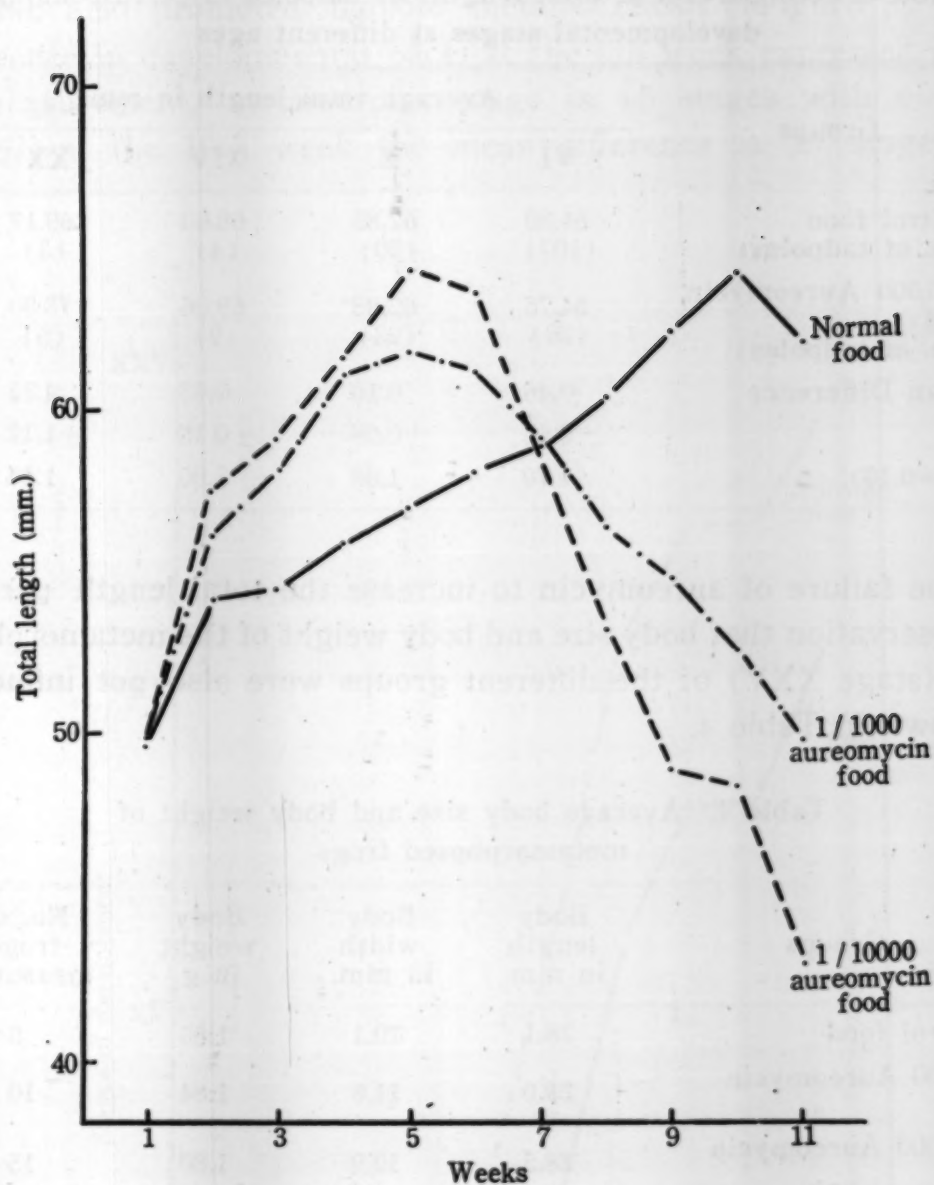


Fig. 2. Growth curves of total length of control and aureomycin fed tadpoles.

A comparison of the average total lengths with respect to different types of food was made by pooling all the tadpoles of the same stage irrespective of their ages. The results are shown in Table 3. It is seen that the mean differences between the total lengths of any stage of the control and the aureomycin-fed tadpoles are not proved to be statistically significant from each other. In other words aureomycin did not affect tadpole total length irrespective of their ages.

Table 3. Comparison of total lengths of tadpoles in corresponding developmental stages at different ages

Groups	Average total length in mm.			
	VI	X	XIV	XX
Control food (No. of tadpoles)	54.30 (107)	62.83 (20)	68.63 (4)	69.17 (3)
1/10,000 Aureomycin food (No. of tadpoles)	54.76 (23)	62.93 (23)	69.06 (9)	73.90 (5)
Mean Difference	0.46	0.10	0.43	4.73
<i>v</i>	± 0.55	± 0.08	± 0.15	± 1.12
<i>t</i> (<i>p</i> =0.10)	1.70	1.68	1.90	1.94

The failure of aureomycin to increase the total length parallels the observation that body size and body weight of the metamorphosed frogs (stage XXV) of the different groups were also not influenced as shown in Table 4.

Table 4. Average body size and body weight of metamorphosed frogs

Groups	Body length in mm.	Body width in mm.	Body weight in g.	No. of frogs measured
Control food	28.1	10.1	1.85	5
1/1,000 Aureomycin food	29.0	11.5	1.84	10
1/10,000 Aureomycin food	28.5	10.9	1.80	15
Mean difference be- tween control and 1/10,000 A. groups	0.4	0.8	0.05	
<i>v</i>	± 0.76	± 0.87	± 0.89	
<i>t</i> (<i>p</i> =0.10)	1.83	1.74	2.02	

The findings indicate that tadpoles fed with aureomycin metamorphosed earlier than the controls, but that their total length and the body size and weight of the metamorphosed frogs were not affected.

II. Aurofac experiment

The results of the second series of experiments indicate that

aurofac also promoted tadpole metamorphosis (Figure 3) (mean difference in developmental pace, at the 6th week expressed in term of measurement, metamorphic stage is 4.5 stages with $v = \pm 5.38$, $t = 1.67$; at the 10th week the mean difference is 3.7 stages with

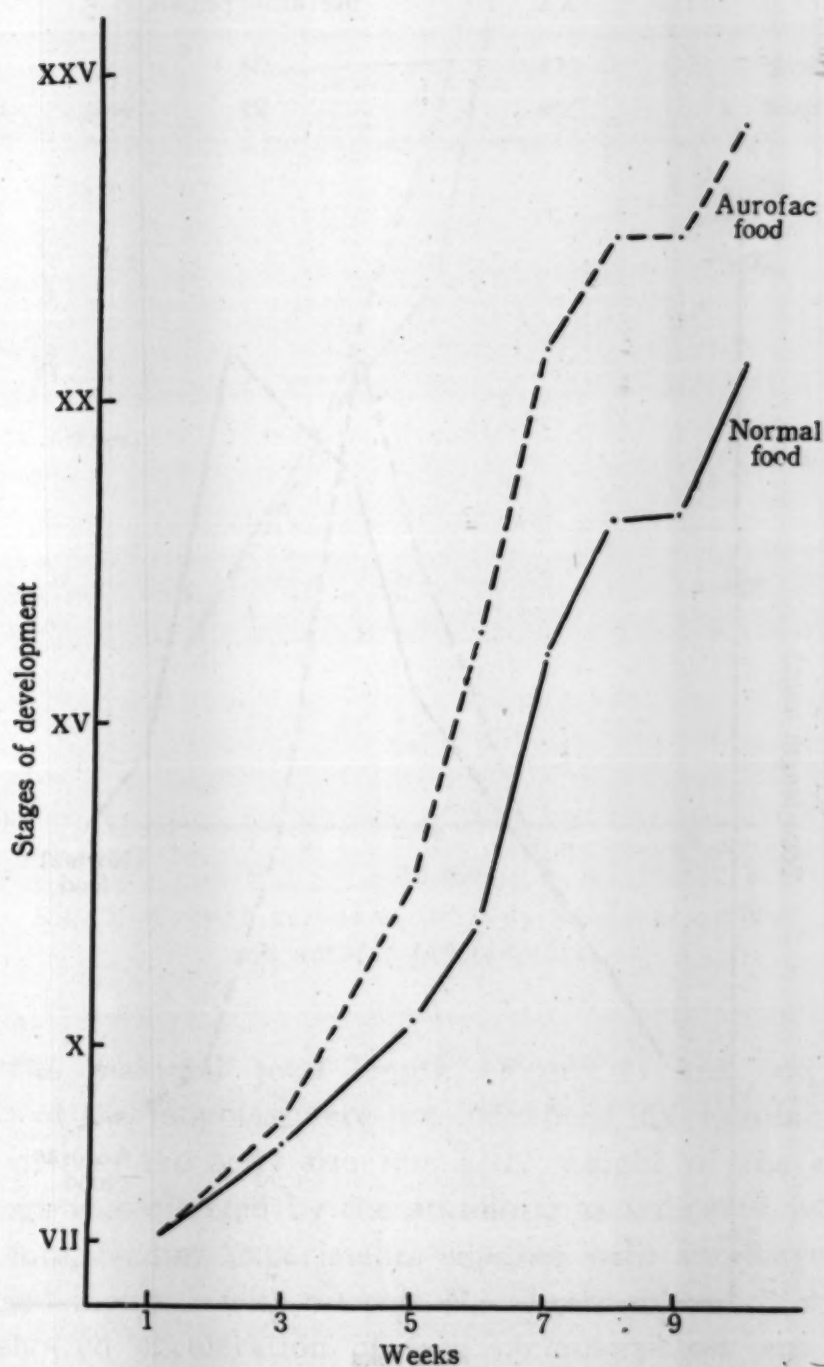


Fig. 3. Growth curves of the metamorphic stages of control and aurofac fed tadpoles.

$v = \pm 3.71$, $t = 1.69$ where $p = 0.10$). The acceleration effect was again paralleled by the metamorphic percentage as is shown in Table 5.

Table 5. Metamorphic percentage
(within a period of 152 days after fertilization)

Groups	No. of tadpoles reaching stage XX	Total No. of tad- poles at end of metamorphosis	% of meta- morphosis
Control food	13	28	46
Aurofac food	29	29	100

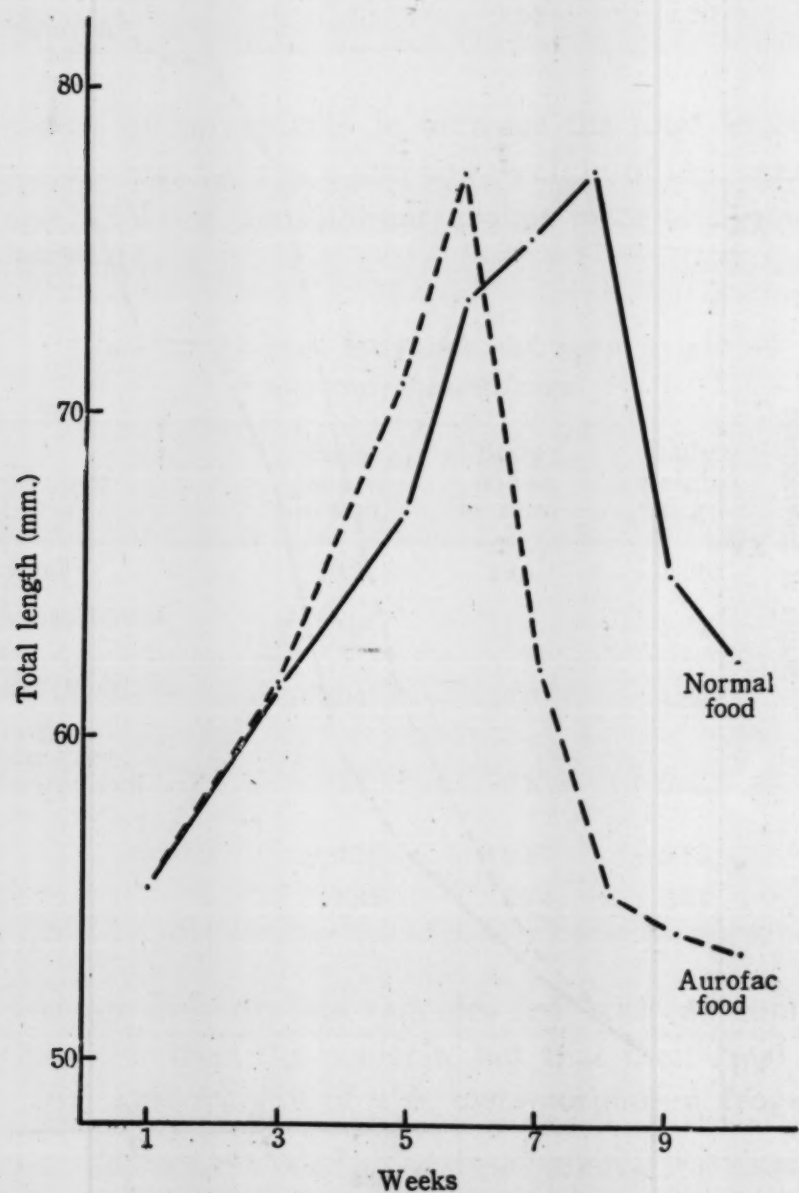


Fig. 4. Growth curves of total length of control and aurofac fed tadpoles.

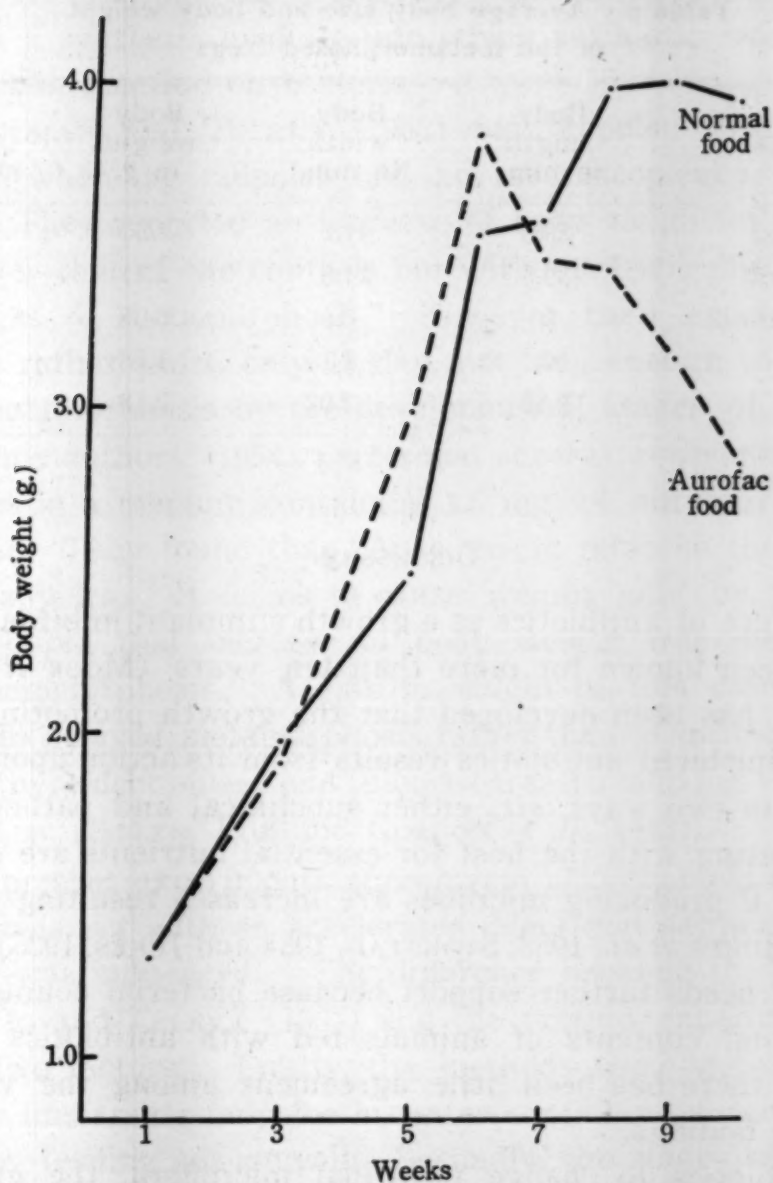


Fig. 5. Growth curves of the body weight of control and aurofac fed tadpoles.

Also the maximal total length (Figure 4) and body weight (Figure 5) of the tadpoles were not influenced by aurofac feeding. Likewise, neither the body size nor body weight of the metamorphosed frogs was affected by the antibiotic as indicated in Table 6.

Therefore, feeding experiments whether with aureomycin in the dosages used together with vitamin B₁₂ or with its crude substitute, aurofac, showed acceleration of frog metamorphosis but did not affect tadpole body size or weight during or at the end of metamorphosis.

Table 6. Average body size and body weight of the metamorphosed frogs

Groups	Body length in mm.	Body width in mm.	Body weight in g.	No. of frogs measured
Control food	29.5	11.2	2.50	4
Aurofac food	29.5	11.8	2.41	27
Mean difference	0	0.6	0.09	
<i>v</i>	0	± 1.15	± 1.78	
<i>t</i> (<i>p</i> =0.10)	2.13	2.02	2.13	

DISCUSSION

The effect of antibiotics as a growth stimulant in birds and mammals has been known for more than ten years (MOOR *et al.*, 1946). The theory has been developed that the growth promoting effect of orally administered antibiotics results from its action upon intestinal microflora in two ways, *viz.*, either subclinical and pathogenic bacteria competing with the host for essential nutrients are suppressed or vitamin B producing microbes are increased resulting in a better nutrition (JUKES *et al.*, 1953, STOKSTAD, 1954 and JUKES, 1955). But this postulation needs further support because bacterial counts made on the intestinal contents of animals fed with antibiotics varied so much that there has been little agreement among the various experimental findings.

If antibiotics do change intestinal microflora, the growth promoting effect would not be observed when antibiotics are administered parenterally. WHITEHILL *et al.* (1950) found no response in chick growth after intravenous injection of penicillin while others (ELAM *et al.*, 1951) reported increased growth in chicks injected with penicillin. However, HESTER *et al.* (1954) showed that injection of aureomycin in dairy calves caused an increase of growth but no aureomycin was found in the lumen of the small intestine. This is antagonistic to the current theory of antibiotic action on growth promoting effect through the intestinal microflora.

NICKELL (1952) reported a stimulation of plant growth in virus tumor in tissue culture, in germinated seeds and in germinated seeds with subsequent growth in soil by the treatment of antibiotics. It

is unlikely that the increased growth value of virus tumor tissue, cultured in a synthetic medium into which antibiotic was added, is due to antibiotic action on bacteria.

MUSTAKALLIO and TELKKÄ (1953) fed tadpoles with liver and aureomycin when the tadpoles had no macroscopically discernible hind legs. They reported an increase of body weight of the treated tadpoles over that of the controls but without observable differences in the stages of metamorphosis. However their experimentation period was rather short, only 15 day, not long enough to judge the speed of metamorphosis by the developmental stages of hind legs.

The same authors (1954) performed another experiment by rearing tadpoles in a medium containing 1.5 mg. of aureomycin in 100 cc. of water. They found that "Aureomycin retarded the metamorphosis of tadpoles. It seems to cause weight gain by this way". They considered that increase of body weight was secondary to retarded metamorphosis. A gain in weight in this case could be attributed to delayed metamorphosis rather than to antibiotic growth effect, for thyroidectomized and thiourea-treated tadpoles would grow to a huge size (ALLEN, 1918 and GORDON *et al.*, 1943).

In the present experiments aureomycin also changed the rate of metamorphosis but with an accelerated effect and neither body size nor weight was influenced. The difference between the results of MUSTAKALLIO and TELKKÄ (1954) and the present experiments may be due to two factors. Firstly, the methods used were different; one was by immersing tadpoles in water containing aureomycin and the other by feeding aureomycin. Secondly, the stages when aureomycin was applied were not the same; in one experiment the antibiotic was applied before the onset of metamorphosis and in the other after the onset. Furthermore D'ANGELO *et al.* (1941) found that retardation or acceleration of tadpole metamorphosis depended on whether starvation was undergone before or after the beginning of metamorphosis. So the same method applied at different times may produce different results.

Amphibian metamorphosis is known to be the result of an integrated action of waves of hormonal activities of pituitary and thyroid glands on tissues of different sensitivities. Therefore MUSTAKALLIO and TELKKÄ's results as well as the present results could indicate that in frog development aureomycin might play a role on

growth not through intestinal microflora but possibly through the pituitary and/or the thyroid.

However the addition of vitamin B₁₂ to aureomycin food in the present experiments seemed to complicate the interpretation of the result. The acceleration of metamorphosis could be attributed to aureomycin or vitamin B₁₂ or both. But in view of the following experiments, vitamin B₁₂ seems to have no effect on thyroid gland. MEITES (1950) found no significant effect on thyroid weight or uptake of I¹³¹ when vitamin B₁₂ was fed to immature rats and thus concluded that vitamin B₁₂ did not alter normal thyroid activity in rats. MUSTAKALLIO and TELKKÄ (1954) noted that vitamin B₁₂ in the dose of 1.5 mg. per 100 cc. of water had no apparent effect on metamorphosis when tadpoles were reared in this medium.

Moreover in the first series of the author's experiments, there were two kinds of aureomycin concentrations but only one level of vitamin B₁₂ in the food for experimental tadpoles and yet the growth curves of the experimental tadpoles showed different rates of development as shown in Figure 1. Should vitamin B₁₂ and not aureomycin be involved in metamorphosis, the growth curves of the treated tadpoles ought to be the same.

On the other hand the relation between aureomycin and endocrine function is rather intriguing. BARBER and coworkers (1953) found the additive effect on growth and feeding efficiency when aureomycin in combination with diethylstilbestrol and *l*-thyroxine was fed to growing pigs. They considered aureomycin as a kind of endocrine stimulants as promoters of growth in fattening pigs. GRANT (1954) reported a small but definite increase of the uptake of I¹³¹ by the thyroid when the antibiotic was fed to rats.

Therefore the accelerated metamorphosis of the present experiments is probably due to some unknown action of aureomycin on thyroid and/or pituitary. The histological comparison of pituitary and thyroid glands between control and experimental tadpoles as well as further experimentation concerning aureomycin and endocrine function is being undertaken, and will be published later.

SUMMARY

Tadpoles of *Rana catesbeiana*, raised from artificially inseminated

eggs, were fed with normal control food and food containing 1/1,000 or 1/10,000 aureomycin supplemented with vitamin B₁₂ beginning at metamorphic stages IV and V. The results showed that metamorphosis of the experimental tadpoles was accelerated at a statistically significant rate when compared with the controls. However, the maximal total length of the tadpoles and the body size and weight of the newly metamorphosed frogs were not influenced. The experiment was repeated with food containing aurofac and same results were obtained. The possibility of altering hormonal activity by this antibiotic is discussed.

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Errata
for
Embryologia Vol. 5, No. 2

p. 201, in Explanation of Fig. 3, line 2/3,
for head neural fold read trunk neural fold

p. 202, in Fig. 4, designation of the 2nd
transverse column,
for ^{e3}
(Intestine) read ^{e3}
(Pr. intestine)

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